Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 105 NOVEMBER, 1960 No. 2

SECTION MEETINGS

DISTRICT OF COLUMBIA
Georgetown Univ. Med. School October 6, 1960

Trachoma Viruses Isolated in the United States* 2. Assay of Egg Infectivity and Stability. (26056)

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Current virologic activity in the field of trachoma was started by T'ang *et al.*(1) who succeeded in growing Chinese strains of trachoma virus and studying their characteristics. The methods of these investigators were rapidly confirmed and trachoma viruses have now been isolated from patients in many parts of the world(2,3,4,5,6,7). However, there have been discrepant reports on the reliability of egg infectivity assays. T'ang(1) obtained regular and reproducible egg LD_{50} values, but Collier and his associates(8,9) stated that "the infectivity of the inocula used cannot be given in terms of 50% end-points" because egg titrations gave very variable results.

When we began to isolate trachoma viruses from patients in the United States(6,7) it seemed important to evaluate the reproducibility of infectivity titrations of our isolates. We wish to record here such observations, together with some stability characteristics of these viruses.

Materials and methods. Viruses: Three representative strains of trachoma virus isolated in the United States(7) were employed. To evaluate possible strain-specific characteristics, pools from 2 yolk sac passage levels (YS) of each isolate were employed, with the following egg LD_{50} titers: Strain BOUR, YS 10 ($10^{-6.4}$), YS 12 ($10^{-4.4}$), strain ASGH, YS 7 ($10^{-7.4}$), YS 8 ($10^{-6.7}$), strain APACHE #1, YS 7 ($10^{-6.7}$), YS 8 ($10^{-5.8}$).

Preparation of stock virus: Virus dilutions estimated to kill 50% of eggs in 6 or 7 days were inoculated in 0.5 ml volume into yolk sacs of 7-day embryonated eggs. Eggs were incubated at 35°C and candled twice daily. Deaths occurring during the first 48 hours of incubation were discarded. When 50% of the inoculated eggs had died, remaining live eggs were refrigerated for 3 hours. Yolk sacs were then harvested aseptically, pooled, weighed, vigorously shaken with glass beads for 5 minutes, and diluted with sterile broth

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* Supported by grants from Nat. Inst. Health, Burroughs, Wellcome Fd. and Res. Comm. of Univ. California.

TABLE I. Sample of Egg Infectivity Titration: Trachoma Strain Asgh YS 7; Jan. 27, 1960.

Virus dilution	No. eggs inoc.	No. nonspecific deaths (48 hr)	Specific deaths on day	Specific deaths Total
10^{-3}	5	1	5, 6, 6, 6	4/4
10^{-4}	5	0	6, 7, 7, 7, 8	5/5
10^{-5}	5	2	8, 9, 9	3/3
10^{-6}	5	0	8, 9, 10, 11, 12	5/5
10^{-7}	5	1	9, 9, 9	3/4
10^{-8}	5	0	11	1/5

$LD_{50} = 10^{-7.4}$.

(Difco proteose-peptone #3) containing 4 mg/ml streptomycin to make a 50% suspension. This stock suspension was distributed in screw-capped vials and stored at –40°C in a mechanical deep freeze.

Infectivity titrations: A vial of stock virus was rapidly thawed, 10-fold dilutions were prepared in broth and kept in an ice bath until inoculated. Five-tenths ml of each dilution was injected by the yolk sac route into groups of 7-day embryonated eggs, incubated at 35°C and candled twice daily. Deaths occurring during the first 48 hours were discarded as non-specific. All deaths from the third to the twelfth days were recorded. Random yolk sacs from dead eggs in each group were smeared and stained by Macchiavello's method to confirm microscopically presence of virus. Such virus was seen regularly in eggs dying on or after the 6th day of incubation. Deaths between day 3 and 5 of incubation were exceedingly rare, and in them virus was seen infrequently. Fifty percent death endpoints were calculated by accepted methods(10) and expressed as the infective titer per g of yolk sac.

Results. After initial isolation in eggs, our strains required several additional passages before infective titers were uniformly high. After being "established" in eggs, infectivity titrations appeared to be reproducible. A representative titration (Table I) shows that the distribution of deaths is proportional to dose. In groups of eggs receiving more than 50 LD_{50} there were rarely any survivors. Repeated assays were performed with a single pool of virus stored at –40°C for 5 months as a 50% yolk sac suspension in broth (Table II). Using only 5-6 eggs per 10-fold dilution, the range of LD_{50} values ($10^{-6.7}$ to $10^{-7.5}$) and range of average days of death for the 10^{-5} dilution (7.0 to 8.7 days) was quite constant for the 8 titrations performed between Jan. 20 and April 6. Subsequently slightly lower titers were found, perhaps because of true loss of infectivity upon prolonged storage at –40°C. Pools of other trachoma viruses have given similarly constant results.

When psittacosis viruses are titrated in eggs, time of death is inversely related to the infective virus inoculum and if sufficiently large groups of eggs are employed, infectivity assays may be performed by observing the average day of death for a single virus dilution(11). When groups of 50 to 100 eggs were inoculated with 10^3 to $10^{4.4}$ egg LD_{50}/ml of stock trachoma virus in preparation of pools, 6-7 days elapsed before 50% of the eggs had died, with variation up to 24 hours. We have not used sufficiently large groups of eggs to establish the "average day of death" for trachoma viruses with great precision. Fig. 1 shows average day of death of small groups (5-6 eggs) inoculated with varying doses of 3 strains of trachoma viruses. It is apparent that in general the linear inverse relationship between amount of virus and time of death applies to trachoma viruses. When average day of death for different virus concentrations was calculated separately for the 2 strains used in most tests the averages fell on straight lines (Fig. 1). It is remarkable that at any dose level, eggs inoculated with strain BOUR died, on the average, one day

TABLE II. Repeated Assay of Egg Infectivity of Trachoma Virus Stored at –40°C.

Date	Egg LD_{50} of stock	Avg day of death of eggs inj. with 10^{-5} dilution
Jan. 20	7.1*	7.5
" 27	7.4	8.6
Feb. 15	7.3	8.2
" 25	6.8	7.9
March 9	7.4	8.7
" 22	6.7	7.6
" 30	7.5	8.4
April 6	7.5	7.0
" 28	6.3	10.4
May 18	6.2	8.6

* Neg. log of dilution of 1 g YS which resulted in death of 50% eggs.

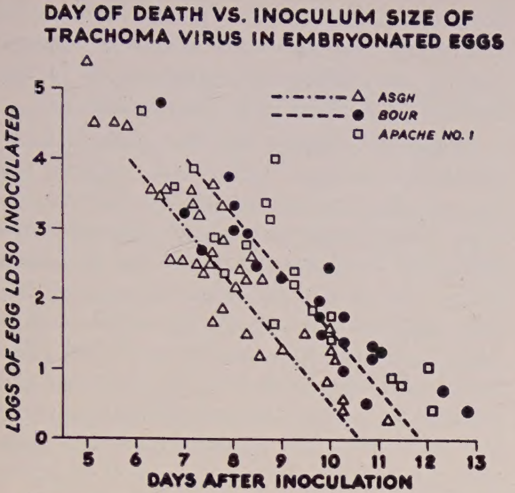

FIG. 1. Relationship of amount of trachoma virus to time of death of inoculated eggs. Each point is observed avg day of death in a group of 5-6 eggs, inoculated with a given amount of one of 3 strains. Lines are fitted to avg day of death calculated from many experiments with each of 2 strains.

later than those inoculated with strain ASGH. This suggests that strain ASGH grows more rapidly to reach a lethal concentration of virus.

Stability. Temperature: Julianelle observed marked temperature lability of infective trachoma virus in the form of fresh conjunctival scrapings(12). T'ang found that in 10% yolk sac suspensions virus was inactivated by incubation at 50°C for 30 minutes or at 37°C for 24 hours, but survived at 4°C for at least 9 days. We stored aliquots of pools of 2 strains of trachoma virus (50% yolk sac suspension in broth, pH 7.1) for various periods at 4, 22 and 37°C and compared the titer by simultaneous titration with that of the same pools stored at –40°C. Loss of infectivity is shown in Table III. Some virus activity survived heating at 60°C for 1 minute, but not for 5 minutes. Inactivation of virus proceeded rapidly at 37°C but there was surprisingly little loss of infectivity at room and refrigerator temperatures. At 4°C titers declined by 0.8 to 1.7 logs in 4 days, and by 1.0 to more than 2 logs in 7 days. Storage at –40°C yielded constant titers for at least 3 months (Table II). Two cycles of freezing and thawing 50% suspensions did not reduce infectivity any more than a single cycle. Virus freeze-dried as 50% suspension of yolk sac in skim milk maintained viability when stored at 4°C for at least 5 months, or when mailed without refrigeration to other parts of the world. A large proportion of viable virus was lost during lyophilization and one blind passage of the freeze-dried material was often required before eggs died with abundant virus in smears.

Ether. When yolk sac suspensions (50% in broth) containing $10^{6.2}$ egg infective LD_{50} per ml were mixed with diethyl ether (30% final concentration) and stored overnight at 4°C no viable virus could be recovered in repeated tests.

Sodium desoxycholate: It has been said that bile salts inactivate trachoma virus(12). Sodium desoxycholate 40 mg/ml was dissolved in 0.25 M sucrose and sterilized by autoclaving(13). Various dilutions of this stock solution were mixed with equal parts untreated 50% suspension of infected yolk sac and the mixture incubated with frequent agitation in a 37°C waterbath for 1 hour. The mixture was then chilled, stained smears prepared, and serial dilutions in broth titrated for infectivity. Up to a final concentration of 20 mg/ml, sodium desoxycholate reduced the infective titer from $10^{7.4}$ to $10^{5.8}$. Whereas Macchiavello or Giemsa stained smears of controls or of mixtures containing 1.2 mg/ml desoxycholate (infective titer $10^{-6.5}$) contained abundant elementary bodies none were seen with concentrations of 2.5 mg/ml desoxycholate or higher. Thus there was gross loss of tinctorial properties at concentrations of desoxycholate which interfered relatively little with infectivity(13).

Discussion. The 3 strains of trachoma viruses used in the present work permitted reasonably precise and reproducible

TABLE III. Loss of Egg Infectivity at Various Temperatures. Avg logs egg LD_{50} inactivated.*

	Hr of storage			
°C	2	7	24	48
4	<.5	<.5	.5	.7
22	.5	.5	.6	.7
37	.8	1.9	3.7	5.1

* Difference in infectivity titer (negative log of dilution) of material kept at –40°C and material stored at indicated temperature.

titration of egg infectivity. This work was performed when egg susceptibility was uniform and could not have been done during the season of partial egg resistance which seems to occur during the late summer or fall months(7). Similar partial egg insusceptibility has been noted by others working with psittacosis viruses(14). Apart from this seasonal variation, the mechanism of which is not understood, we were able to obtain reliable 50% endpoints in defining the infectivity of inocula in apparent contrast to the experience of the Lister Institute workers (8,9). The general inverse linear relationship between infective inoculum and time of egg death applied to our trachoma viruses. However, small but definite variations were observed in time of death from a constant inoculum. In spite of this slight heterogeneity in egg response, it was shown clearly that strain ASGH tended to kill eggs more rapidly than strain BOUR, at all inoculum levels. This difference is interesting because these 2 strains differ markedly in other respects, such as severity of experimental disease in monkeys and behavior toward antibiotics. In view of the heterogenous clinical picture of trachoma in various parts of the world such strain differences deserve to be explored further. Maintenance of viability by lyophilization should permit ready exchange of representative strains between different laboratories.

The relative stability of trachoma viruses at 4°C confirms the use of "wet ice" as possible transport medium for specimens(2,8). The rapid loss of infectivity at 37°C parallels other viruses of the psittacosis group. The destruction of our trachoma viruses by ether is in keeping with the ether lability of other psittacosis-like viruses but contrasts with T'ang's statement that "ether . . . possesses no effect on the virus"(1). Sodium desoxycholate interfered with tinctorial properties of the virus far more than with infectivity, in the presence of massive amounts of yolk sac components. While the presence of these protective materials does not permit conclusions concerning the anti-viral effects of desoxycholate, the differential action merits further study with purified virus suspensions(13).

Summary. Except for the seasonal insusceptibility of eggs during late summer and fall, reliable and reproducible infectivity assays were performed on 3 representative strains of trachoma virus isolated in United States. In general the linear inverse relationship between amount of virus and time of death applied to these trachoma viruses. One strain (ASGH) tended to kill eggs one day earlier than another strain (BOUR) at all inoculum levels. The strains were ether labile, and rapidly lost infectivity at 37°C or above. Sodium desoxycholate interfered with staining properties of virus far more than with infectivity.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Influence of Certain B-Vitamin Antimetabolites on Ovulation *in vitro* in the Frog, *Rana pipiens*.* (26057)

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Induction of ovulation in a typical vertebrate classically requires cooperation of hypophyseal FSH and LH to rupture follicular walls and effect release of ova(1). In frogs, where *in vitro* procedures can be used conveniently, evidence indicates that glycolysis, tricarboxylic acid cycle, and cytochrome-cytochrome oxidase systems are essential for ovulation and that reactions leading to extrusion of ova are endergonic(2). Various steroids appear to play a supporting role in ovulation (3,4) and may even substitute for pituitary factors entirely(5). The foregoing information as well as experiments using unfractionated frog pituitary extracts and mammalian FSH and LH(6,7) suggest that *sensitization* of ovarian tissue may be a distinct process from *ovulation itself*. Data of this communication, obtained using certain antimetabolites in connection with ovulating fragments of excised frog ovary, also support a concept of sensitization as separate from ovulation.

Methods. Ovaries and pituitary glands were dissected from Vermont frogs shipped to the laboratory at frequent intervals. Preparation of pituitary extracts consisted of grinding several fresh glands at a time in a small mortar, lysing any remaining cells with a small quantity of distilled water, and diluting to desired concentration with standard Ringer's solution. Ovaries were cut into lobes and placed at random in Petri dishes with 50 ml Ringer's fluid containing pituitary extract and any vitamin or antimetabolite to be tested. Numbers of eggs ovulated were recorded after 22-24 hours at 22-24°C. Possibility of spontaneous ovulation (*i.e.*, without added pituitary stimulation *in vitro*) was checked by placing ovarian lobes in plain Ringer's fluid (Ringer controls), but in no case were any ova extruded without pituitary influence. Standard responsiveness of each ovary was determined by exposing one lobe to a concentration of from 0.1 to 0.5 pituitary gland (pituitary controls), exact dosage depending on the season. Responses to identical dosages of pituitary may vary considerably from animal to animal, undoubtedly due to unknown and immeasurable differences in physiological state. Unless pituitary controls extruded at least 15 eggs upon optimal stimulation, sensitivity of ovarian tissue was not considered sufficient to provide reliable data.

Results. Preliminary tests showed (Table I) that addition of B vitamins to ovulating media neither enhanced nor impaired responses to pituitary extract. In experiments run during December (Table IIA), antimetabolites of nicotinic acid (*α*pa, p3sa, and acetyl pyridine) markedly inhibited ovulatory responses to pituitary extracts. About 25% of this inhibition could be removed by simultaneous inclusion of $5\mathrm{x}10^{-4}$ M nicotinic acid. In contrast, antimetabolites of thiamine (oxythiamine), biotin (desthiobiotin), folic acid (aminopterin), pyridoxine (desoxypyridoxine) and pantothenic acid (pantoyl taurine) showed no inhibition of ovulation. From these data it appears that metabolism of nicotinic acid is in some way intricately involved in ovulation, but that other B vitamins are not directly implicated.

During April, *pipiens* ovary is at its peak of sensitivity, and a retest of nicotinic acid

TABLE I. Effect of B Vitamins on Ovulation *In Vitro.*

Vitamin (5×10^{-4} M)	No. of exp.	No. of eggs released in Pituitary controls	No. of eggs released in Pituitary + vitamin
Nicotinic acid	4	173	163
Thiamine	5	265	278
Biotin	2	76	80
Folic acid	7	271	306
Pyridoxine	3	142	130
Calcium pantothenate	3	51	43
Inositol	4	187	199
Riboflavine	6	303	290

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* Aided by a grant from Nat. Inst. Health.

TABLE II. Effect of B-Vitamin Antimetabolites on Ovulation *In Vitro*.

		No. of eggs released in			
			Pituitary +		
Antimetabolite	No. of exp.	Pituitary controls	.001 M antimet.	.0005 M antimet.	.0001 M antimet.
A. December Exp.					
α-Picolinic acid (αpa)	5	329	1	83	117
Pyridine-3-sulfonic acid (p3sa)	6	439	0	1	34
Acetyl pyridine	4	167	2	30	73
Oxythiamine	2	57	74	65	56
Desthiobiotin	2	196	194	166	208
Aminopterin	2	106	117	105	113
Desoxypyridoxine	2	91	80	99	94
Pantoyl taurine	4	130	124	125	136
B. April Exp.					
α-Picolinic acid	6	253	244	—	291
Pyridine-3-sulfonic acid	4	361	213	—	303
Acetyl pyridine	3	140	142	—	187

analogues at this time revealed a lack of inhibition for αpa and acetyl pyridine, and a much reduced inhibitory activity for p3sa (Table IIB). A retest of antimetabolites of other B vitamins at this time confirmed our December results.

Discussion. It is not surprising to find that ovulation was impaired during winter months by analogues of nicotinic acid, for it is now well known that DPN plays an important and almost ubiquitous function in release of cell energy. In early winter most frog ovarian tissue is only moderately sensitive to pituitary factors, and intermediate to large dosages are still required for maximal ovulation. In April, nicotinic acid analogues had lost most or all of their ovulation-inhibiting capacity. This is the breeding season for this species in the wild, and only minimal dosages of pituitary extract are required for optimal ovulatory response of laboratory animals at this time, either *in vivo* or *in vitro*. Between December and April, then, a sensitization of ovarian follicles has occurred so that ovulation may proceed not only more rapidly but also in response to smaller dosages of ovulatory factors. From data provided above, it appears that nicotinic acid or DPN is required during winter months while sensitization of follicles proceeds, prior to actual ovulation and during the time that FSH is exerting its principal effect. Metabolism of nicotinic acid or DPN appears to be unnecessary in ovulatory reactions induced in April after sensitization has presumably already occurred. Time studies(8) bear out this same scheme, since beginning of ovulation *in vitro* in early winter may require 14-18 hours but only 6-8 hours in April. Difference in time may well be explained by a necessity for 8-10 hours of sensitization time in winter, and absence of this requirement in spring.

Summary. Ovulation *in vitro,* as induced by pituitary extracts, was inhibited by antimetabolites of nicotinic acid in December experiments but not in April. Results suggest that metabolism of nicotinic acid may participate in sensitization of frog ovary which occurs gradually during the hibernation months from December to April, but not directly in ovulation itself. Other B vitamins were not implicated.

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Received July 27, 1960. P.S.E.B.M., 1960, v105.

Direct Determination of Systemic Blood Pressure and Production of Hypertension in the Rabbit.* (26058)

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Repeated determinations of the blood pressure of the unanesthetized rabbit have been carried out in the past mainly by the indirect method of Grant and Rothschild(1), by means of an airtight capsule, applied to the ear, which permits visualization of the central artery. The pneumatic pressure required just to break the column of blood in this portion of the central artery, in systole, is taken as the systolic pressure in the artery. This pressure is lower than the direct mean pressure in the common carotid artery and is regarded by Grant and Rothschild merely as an "index" of systemic blood pressure. A number of disadvantages have interfered with the general use of this method, hence the rabbit has not been employed frequently in studies on experimental hypertension(2-5). One great drawback of this method is that the central artery must be in a state of dilatation, or at least not in constriction, for valid observations to be made, because a constricted artery gives lower values, and it is sometimes impossible to make a determination if the artery is greatly constricted. For this reason recent denervation of the ear is desirable, to avoid great fluctuations of the calibre of the central artery, which greatly modify the determination. If the ear is not denervated, or if the central artery has recovered from the effects of denervation, which often occurs within a few days, then to avoid changes in the calibre of the central artery, it is necessary to make all measurements in a very warm room, or to contrive to heat the whole rabbit, or at least the ear. It is also important always to apply the capsule to the same part of the central artery, because a variation of a few centimeters, toward the base or toward the tip of the ear, means a corresponding considerable increase or decrease, respectively, of blood pressure in the central artery.

Recently, during another study, it became necessary to use hypertensive rabbits, with repeated determinations of blood pressure of unanesthetized rabbits before and after surgical procedure for production of hypertension. Because of the drawbacks of the capsule method, it was desirable to develop a method of taking the blood pressure of the conscious rabit without carrying out determinations in a hot room, or heating or denervation of an ear. Having found the direct method reliable in determination of mean blood presure in the femoral artery of many unanesthetized dogs and other large animals(6), I decided to try this method on large rabbits.

Method for determination of blood pressure. Rabbits of different varieties, of both sexes, weighing from 3.5 to 5 kg were used. For direct determination of blood pressure in the femoral artery, the unanesthetized animal is placed in the supine position on a wooden trough, with both hind legs restrained lightly just above the ankles by wide leather straps tied to the sides of the board. The front legs may also be tied down, or preferably, an assistant[†] may hold the front legs and pull the animal up slightly to stretch the femoral artery somewhat. Once the animal is fixed and held on the board it usually remains remarkably quiet. It requires some skill, but with practice it is possible regularly, even several times daily if necessary, to insert a 22 gauge needle of ¾″ length through the skin into a femoral artery of a large unanesthetized rabbit. The needle is on the end of a glass cannula connected by plastic tubing to a mercury manometer of

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* This investigation was supported by Research Grants from Nat. Heart Inst. and L. D. Beaumont Foundation.

[†] I wish to acknowledge with my sincere thanks the help of Mr. J. Malone.

capillary bore. A large bottle containing normal saline solution, which can be subjected to pressure, communicates with the mercury manometer and with the tubing which leads to the needle. Manipulation of 2 stopcocks permits elevation of the pressure in the manometer and flushing of the glass cannula and needle. With the tip of the index finger of one hand in the groin, just below Poupart's ligament, the pulsating femoral artery is located, and this finger acts as a guide for the tip of the needle after it has been inserted through the skin and subcutaneous tissue. The needle is manipulated by the other hand which holds the glass cannula to which the needle is attached. It is important to use a sharp needle, to work slowly, and to push cephalad, gently, until the tip of the needle enters the artery. Successful puncture of the artery is signaled by the prompt entrance of a small amount of bright red blood into the cannula. If connection with the manometer is then established, by turning a stopcock, definite pulsation can be observed. The vein is so close to the artery, however, and both are of such small calibre, that occasionally the vein is punctured. This is easily recognized, because the blood is darker than arterial blood and because, if the stopcock is turned so as to connect the cannula with the manometer, the pressure in the manometer is very low and there is no pulsation. Puncture of the anterior crural nerve must be avoided and rarely occurs if the position of the nerve is kept in mind. After determination of the blood pressure has been made, the needle is withdrawn and pressure is applied at the site of puncture for a minute or more to prevent bleeding from the puncture in the skin or formation of a subcutaneous hematoma. If one does form, it may be expected to disappear in a few days, and the artery may then be punctured again at the same site. Clotting rarely occurs during the brief period of observation of blood pressure because, by proper arrangement of the 2 stopcocks in the system, it is possible to raise the level of the mercury in the manometer and flush out the cannula and needle. Neither thrombosis nor infection at site of puncture has occurred in any of the animals, although the skin is not sterilized and the needle, though clean, is not sterile.

TABLE I. Direct Mean Femoral Blood Pressure, mm Hg, of 24 Normal Rabbits Weekly for 3 Weeks.

Rabbit No.	Initial B.P.	8th day	15th day	22nd day
1	102	106	108	108
2	98	98	102	102
3	100	104	110	110
4	92	98	90	90
5	112	108	104	104
6	94	92	104	104
7	114	114	102	106
8	114	110	100	106
9	116	105	110	106
10	108	110	110	110
11	102	105	102	108
12	108	106	106	102
13	108	114	110	112
14	106	112	110	106
15	108	104	106	94
16	122	120	116	128
17	124	122	124	122
18	106	112	108	110
19	122	114	120	126
20	92	98	102	100
21	96	98	100	98
22	118	116	124	126
23	122	110	118	120
24	102	106	100	110

Blood pressure of normal rabbits. Table I gives direct mean femoral blood pressure, taken once a week, for 3 weeks, of 24 large, normal rabbits, of both sexes (mainly females), and different breeds (Chinchilla, New Zealand White, American Blue, Brown or Sandy), weighing from 3.3 to 5 kg. The relatively small variation from week to week in individual animals, and the slight differences between the animals are very striking. Table II shows blood pressures for 12 weeks of 3 female and 3 male Chinchilla rabbits weighing between 4 and 5 kg. In both Tables I and II the values are much more uniform than those that have been obtained by the indirect method of Grant and Rothschild(1-5).

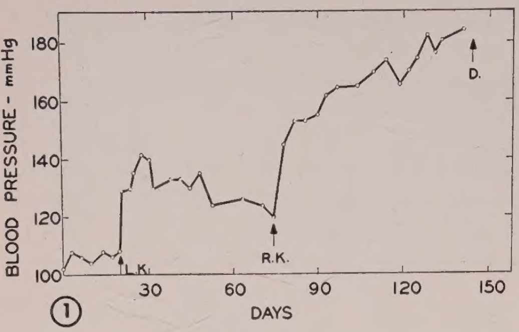
Production of experimental hypertension in the rabbit. Surgical procedure. The method for production of hypertension used in this study was constriction of both main renal arteries, or constriction of one main renal artery and contralateral nephrectomy(6). Because the diameter of the main renal artery of a rabbit weighing from 3.5 to 5 kg is so small, the type of silver clamp which was devised for constriction of the much larger arteries of

TABLE II. Direct Mean Femoral Blood Pressure, mm Hg, of 6 Rabbits Weekly for 12 Weeks.

Rabbit No. and sex	4/18	4/25	5/2	5/9	5/16	5/23	5/31	6/6	6/13	6/20	6/27	7/6	7/11
26 ♂	120	116	110	106	110	112	116	116	110	112	106	98	106
27 "	104	102	110	106	108	100	100	96	96	102	98	96	96
28 "	108	98	106	106	110	98	104	102	104	100	94	96	98
29 ♀	104	110	110	106	108	106	114	98	110	108	110	98	106
30 "	108	110	114	114	122	114	112	118	110	106	110	96	110
31 "	100	100	102	104	98	102	108	110	96	108	100	94	100

dogs weighing from 15 to 20 kg was not employed. The fashioning of a very small silver clamp of this type is difficult and too expensive. A silver ribbon clip was employed, of the type used for the rat(7), but made from a silver ribbon 0.25 cm in width and 0.8 cm in length. The ribbon is bent at its middle over a round wire 0.15 cm in diameter and one free edge is turned up slightly to facilitate handling with forceps. As in the dog, for isolation of a part of the renal artery, the retroperitoneal approach is used, and the operation is carried out through an oblique lateral abdominal incision, just below and parallel to the costal margin, beginning at the costolumbar angle. The muscles are split in the direction of their fibers and, during splitting of the transversalis muscle, special care is taken not to puncture or tear the peritoneum which is stripped away from the lumbar muscles by means of a finger tip. A small self-retaining retractor is then inserted through the wound. The kidney and main renal artery are easily identified. The first part of the main renal artery is dissected free and cleaned for about 1 cm or less from its origin in the aorta. The artery is lifted by means of a metal hook into the rounded part of the clip and is then completely enclosed in the clip by pinching together the free edges. For production of the desired degree of constriction of the artery by the silver clip, a special forceps devised in our laboratory for the rat(8) has proved useful. By means of this forceps degree of constriction of the artery can be varied at will. Once the vessel is enclosed in the clip, the entire clip is squeezed in the jaws of the forceps to the limit permitted by the previous setting of the screw for separation of the jaws(8).

Results of constriction of one or both main renal arteries. In some of the rabbits the degree of constriction proved too great, so that the kidneys became partly or completely necrotic. When this occurred bilaterally, fatal uremia, usually accompanied by hypertension, quickly developed. In other animals, when the arteries were not adequately constricted, there was no elevation of blood pressure. By trial and error, however, it was found that a gap of 0.030″ to 0.032″ between the jaws of the forceps gave the right degree of constriction of the main renal artery of a rabbit weighting between 3.5 and 4 kg, while 0.034″ to 0.036″ was best for a rabbit weighing between 4.5 and 5 kg. Fig. 1 and 2 show the blood pressure records of 2 rabbits before and after constriction of first one and then the other main renal artery. Peart(9) has stated that, "without an elevation of blood urea no elevation of blood pressure occurs in rabbits." This was undoubtedly true for his rabbits, but that it is not applicable to all rabbits, is shown by Rabbit No. 25, (Fig. 2), in which blood urea, creatinine and non-protein nitrogen remained well within the limits of normal throughout the entire period of the considerably elevated blood pressure. The reason for the occurrence of impairment of renal excretory function in the rabbit more frequently than in the dog is that it is more difficult to avoid excessive constriction of the main renal artery in the rabbit, and uremia is especially likely to occur if the artery of one kidney is constricted and the other kidney is removed. This is a good method for production of persistent hypertension, in the dog, but hazardous in the rabbit in which it is better to constrict both main renal arteries, with an interval of a week or more between operations. Fig. 1 and 2 illustrate the results of the latter procedure in 2 rabbits. In



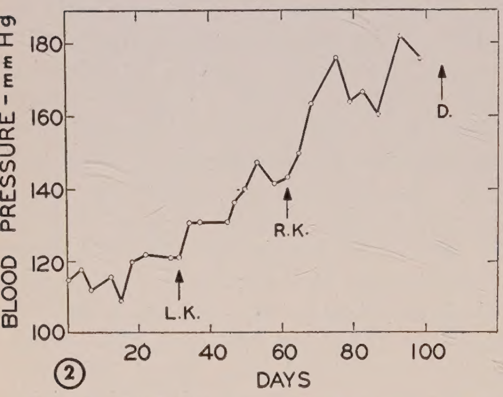


FIG. 1. Rabbit No. 1. L.K., moderate constriction of left main renal artery. R.K., moderate constriction of right main renal artery.

FIG. 2. Rabbit No. 25. L.K., moderate constriction of left main renal artery. R.K., moderate constriction of right main renal artery.

Rabbit No. 1 (Fig. 1) blood pressure was returning to the original level before the right main renal artery was constricted, 8 weeks after constriction of the left, while in Rabbit No. 25 (Fig. 2) blood pressure was still rising when the right was constricted 30 days after constriction of the left main renal artery. In both animals blood pressure rose appreciably after constriction of the first main renal artery, but became much more persistently elevated, after constriction of the second. Both animals died unexpectedly, and the only abnormalities found at autopsy were dilatation of the chambers of the heart and moderate edema of the lungs. In Rabbit No. 1 (Fig. 1) no chemical determinations of the blood were made, but in Rabbit No. 25 (Fig. 2) blood urea nitrogen, creatinine and non-protein nitrogen values remained unchanged after constriction of both arteries and development of persistently elevated blood pressure. Other hypertensive rabbits have also failed to show significant alteration of renal excretory function.

Summary. A method is described for determination of direct mean systemic blood pressure in the femoral artery of the unanesthetized rabbit. Weekly blood pressures of 24 rabbits, for 3 successive weeks, and of 6 other rabbits, for 12 weeks, are given. Production of persistent renal hypertension, unaccompanied by significant impairment of renal excretory function, is described and illustrated. It is hoped that publication of this method for determination of the blood pressure of the unanesthetized rabbit will stimulate others to employ this animal more often in future studies of experimental hypertension.

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Received Sept. 29, 1960. P.S.E.B.M., 1960, v105.

Observations on Culture *in vitro* of Normal and Dystrophic Muscle Tissue.*†
(26059)

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Efforts to demonstrate reproducible differences *in vitro* in growth patterns of cells from normal and dystrophic human tissue and thus to characterize the dystrophic condition have remained discouraging over the last 5 years of this study. As reported previously(1,2), some of the measured growth parameters were either widely scattered or markedly inconsistent. Other differences, *e.g.*, in DNA contents, which were more consistent were not necessarily characteristic for the dystrophic state of the muscle. Reports by other investigators(3,4) of pathologically significant differences could not be confirmed. Better defined and more uniform tissue samples were obtained from a strain of chicks with hereditary muscular dystrophy, primarily of the breast muscles(5). Quantitative differences in growth pattern of normal and dystrophic chick muscle cells in media with sub-optimal embryo extract concentrations were observed. These differences are statistically significant and may be related to the pathological state of the tissue. The data with chick muscle explants and a brief summary of the older experiments with human muscle are reported here.

Materials and methods. Chick muscle: The chicks, obtained from the flock at Univ. of California, Davis,(5) during the first week after hatching, were raised at our laboratory to an age of 7 weeks. Dystrophic manifestations (inability to stand up when put on back) became apparent during the third week after hatching. The animals were sacrificed at age intervals of 2 weeks by decapitation under ether anesthesia and hypothermia. Pectoral muscles were dissected and minced, 5-10 mg quantities, weighed on a "desicoted" pan, were transferred into roller tubes on a "desicoted" glass spoon and imbedded in a clot composed of .25 ml chicken plasma diluted 1:6 with Pannett-Compton saline(6) and 0.025 ml thrombin diluted 1:4. The nutritional media consisted of 40% horse serum, either 5% or 2.5% embryo extract and 20% synthetic medium (SM, 7) made up, to volume, with Pannett and Compton solution containing 100 mg % glucose, and penicillin and streptomyocin at a concentration of 100 units/ml and 100 μg/ml respectively. Embryo extract was prepared from 12-day chick embryos. Cultures were fed 3 times a week.

Protein nitrogen (PN) and deoxyribonucleic acid (DNA) determinations were made over a 3 week period. DNA was estimated by the Burton method(8). PN values, obtained by Nesslerization, were corrected for PN of the plasma.

Human muscle: A small piece (about 1 cm^3) of gastrocnemius was secured from dystrophic patients with the diagnosis of pseudo hypertrophic dystrophy made by the Pathology Department after examination of biopsies. Normal human skeletal muscle was obtained from patients not afflicted with muscular dystrophy.

Viability tests with human biopsies: The muscle was freed of connective tissue and cut into pieces of about 1 mm^3. Six to 8 pieces were imbedded in a single row in a clot of chick plasma in a roller tube. The medium was composed of 40% human serum, fetal pig brain or muscle extract, and other constituents made up as for the chick medium. The

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* This investigation was supported by grants from Muscular Dystrophy Assn. of America and Inst. of Neurological Dis. and Blindness, N. I. H., U. S. P. H. S.

† The authors are indebted to Drs. V. S. Asmundson and L. M. Julian, Univ. of California, Davis, for supplying dystrophic chicks and for much valuable advice in the work with chick muscle, to the staff of the Dept. of Surgery, Univ. of Colorado Med. School, for supplying the human biopsies, and to Mrs. Dorothy C. Lowry, Univ. of California, Berkeley, for extensive statistical evaluation of data.

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TABLE I. Deoxyribonucleic Acid (DNA) Content of Muscle Explants Expressed as μg DNA/mg of Original Wet Weight of Explants.

Each figure is mean of separate determinations on 6 cultures. N = Normal, D = Dystrophic, EE = Embryo extract.

Period *in vitro*	N	D	N	D	N	D	N	D
Series I			Age of donor chick (days)					
5% EE	4–5		14		21		42	
0	1.8	1.6	.4	1.1	.4	.6	.3	.7
2	1.0	1.0	.3	.6	.2	.3	.1	.3
7	3.4	5.6		2.3	.6	1.5	.7	1.3
14	5.6	8.0	3.3	3.1	1.4	3.0	1.7	2.1
Series IIa			Age of donor chick (days)					
5% EE	7		21		35		49	
0	.7	2.1	.4	1.2	.4	1.1	.4	.7
	.7	2.0	.4	1.1	.3	.7	.3	.7
2	.5	.9	.2	.3	.1	.4	.2	.3
	.4	.6	.2	.3	.2	.4	.1	.2
7	1.2	2.7	.2	.9	.3	1.4	.6	1.9
	1.5	2.5	.4	1.3	.4	1.2	.5	1.1
14	1.0	2.1	.5	.9	1.1	1.6	1.3	1.4
	1.7	1.2	.9	1.2	.7	2.5	1.0	2.7
Series IIb			Age of donor chick (days)					
2.5% EE	7		21				49	
0	.8	2.1	.4	1.2			.4	.7
	.7	2.0	.4	1.1			.3	.7
2	.5	.8	.2	.3			.2	.2
	.4	.7	.2	.4			.1	.3
7	.8	2.0	.2	.6			.4	1.3
	.9	1.7	.3	.9			.3	.7
14	.7	.9	.2	.7			.7	.5
	.9	1.0	.4	.6			.4	.8

In Series I one normal and dystrophic donor animal each was used for each donor age. In Series IIa and IIb 2 normal and dystrophic donor animals each were used for each donor age. Tissue samples in Series IIa and IIb were obtained from the same donor animal and were identical in respect to all culture conditions except extract concentration.

fetal pig extracts were prepared by homogenization of the tissue for a few seconds in a blender with one half the tissue volume/weight of Pannett-Compton solution, centrifuged for 45 minutes at 3,000 × g. The supernatant was diluted (between 1 and 5 times) to contain 2 μg/ml of protein and 0.4 ml of the diluted extract was used in a total volume of 2 ml of medium. Explants were fed twice a week and sub-cultured at 4 week intervals. At this time the outgrowth was usually so extensive that only the tissue from one tube in a series was maintained.

Short term cultures of human biopsies. Biopsy samples were minced and placed into roller tubes similar to the chick muscle. Media containing various proportions of human serum and SM were tested. Addition of embryo extract was found to be unnecessary in the case of human muscle.

Results. Analyses of the explanted chick muscle cultures show (Table I) that beginning with donors of 7 days after hatching the freshly excised muscle samples (O period *in vitro*) from homozygous dystrophic chicks show a higher DNA content than normal controls. In the younger (4-5 day) donors in Series I this difference was not observed. During the first 2 days of culture of normal and of dystrophic muscle, about one half of DNA content of the initial explant is lost, but proliferation from the second to the seventh day of cultivation compensates for this loss. DNA content of either normal or dystrophic muscle showed variable changes during the 7-14 day growth period.

In both types of chick muscle, in contrast to human muscle, there is no loss, but actu-

TABLE II. Protein Content of Muscle Explants Expressed as μg Protein Nitrogen/mg of Original Wet Weight of Explants.

Each figure is mean of separate determinations on 6 cultures. N = Normal, D = Dystrophic, EE = Embryo extract.

Period *in vitro*	N	D	N	D	N	D	N	D
Series I			Age of donor chick (days)					
5% EE	4–5		14		21		42	
0	7.2	5.0	10.3	8.3	10.4	8.2	10.9	11.3
2	10.5	8.4	14.4	10.9	12.4	10.8	13.2	13.1
7	20.8	25.6	15.9	15.5	14.2	15.6	15.0	14.8
14	29.7	37.8	26.1	21.6	18.1	24.2	20.6	16.8
Series IIa			Age of donor chick (days)					
5% EE	7		21		35		49	
0	7.7	5.6	10.2	9.5	9.9	12.7	10.8	11.5
	8.3	8.6	11.3	12.1	8.5	9.3	11.2	10.3
2	11.1	10.5	12.4	11.7	12.2	12.3	13.6	13.1
	12.4	11.2	13.6	12.5	10.5	12.6	13.4	12.8
7	12.8	14.1	14.1	13.2	13.4	12.2	16.2	13.4
	16.3	16.4	15.3	14.1	12.9	16.9	15.7	16.2
14	10.2	14.9	15.7	10.2	16.4	13.0	16.5	12.1
	15.0	10.5	15.2	11.2	13.8	19.7	17.6	21.0
Series IIb			Age of donor chick (days)					
2.5% EE	7		21				49	
0	7.7	5.6	10.2	9.5			9.0	11.5
	8.3	8.6	11.3	12.1			11.2	10.3
2	10.1	9.5	12.5	12.0			12.8	14.0
	11.7	9.9	13.1	13.4			13.0	12.5
7	11.0	11.7	12.4	10.2			14.2	9.2
	13.4	12.0	12.1	11.7			14.9	12.4
14	7.6	7.6	12.3	6.3			12.9	5.8
	8.7	8.0	13.7	5.1			15.0	8.9

Conditions identical to those of Table I.

ally an increase, of protein content during initial culture period (0-2 days, Table II). A variable increase in protein content was observed during the 2-14 day culture period. In one of the series (I), embryo extract at a concentration of 5% leads to large increases in the PN in both normal and dystrophic animals. In another series (IIa) with the same extract concentration, the increases were smaller in cultures of both muscle types. The embryo extract used in the first series was obtained during the height of the laying season. Whether this was the reason for the difference in growth promoting quality of the extract remains undecided.

Replicate cultures of the same muscle samples as those of Series IIa were set up with 2.5% embryo extract (IIb). In this lower concentration of embryo extract, cultures from normal and dystrophic 7-day-old donors showed a decreased protein content during the later culture periods. Cultures from older, normal donors did not show this loss of protein, but in all cultures from the older dystrophic donors a marked decrease during the 2-14 day culture period was observed. Observation of these differences is based on cultures from only 4 donors. A statistical analysis of the figures for individual cultures showed that the probability of this difference occurring by chance was less than 5%. The fact that the samples for Series IIa and IIb were obtained from the muscles of the same donor increases the statistical significance of these findings. Such a result may indicate that maintenance of dystrophic muscle remains dependent on higher extract concentrations than do both muscle types up to the 7th day of donor age. In contrast, maturing normal muscle, after the 7th day of donor age, can maintain its protein content even with the lower extract concentration.

In freshly excised human muscle, just as in chick muscle, DNA contents of dystrophic tissue were found to be higher than in controls. In human tissue, both PN and DNA contents show a transitory decrease reaching a minimum between the second and fourth day of cultivation. After culture for 3 weeks, in a standard medium, both normal and dystrophic muscle reached PN and DNA values about 2 times higher than initial values. If SM was omitted, growth of the cultures in 20% or 40% human serum reached only the initial values. No consistent differences could be found in the growth pattern of normal and dystrophic human muscle in any one of these culture conditions. Cultures from 2 human dystrophic donors grew much slower than controls, in cultures from 3 donors the muscle gave higher DNA and PN values than controls, and in 4 series no significant difference could be detected.

In further tests with human muscle attempting to repeat the experiments of Geiger and Garvin(3,4) neither the number of cultures showing outgrowth after one month nor the maximal duration of *in vitro* maintenance revealed striking differences between growth characteristics of normal and dystrophic muscle tissue. It became apparent that, for no obvious reasons, viability of roller tube cultures varies greatly and that a statistically valid establishment of possible differences in onset of rate of growth would presuppose measurements on very large numbers of cultures under these conditions. In our series of 5 normal muscle samples, maximal survival periods for cultures from different individual donors varied from 3 to 6 months, and in the cultures from 8 dystrophic biopsies from 2 to 11 months. In cultures of normal muscle 67% to 97% survived the first month; in dystrophic muscle 42% to 98%.

Cell types obtained during cultivation of both normal and dystrophic muscle were of a wide morphological variety. They included fibroblast-like cells and sarcoblasts which interlaced to form a dense meshwork. Sometimes the cultures were surrounded by giant cells containing many nuclei and vacuoles. These cells are probably the same as those described by Geiger and Garvin(3,4) as specific for dystrophic muscle. However, in our investigation, they were found in both normal and dystrophic explants and usually indicated imminent death of the culture.

Discussion. The results of the measurements on chick muscle cultures suggest that normal and dystrophic cells grown *in vitro* may differ at least quantitatively in their requirements for a factor which is supplied by embryo extract. Confirmation of existence of such a difference would concentrate the search for the nature of the defect of the dystrophic cell and for factors which eliminate this defect on a well limited area of materials and technics. Harris and Kutsky(9) have recently isolated the protein of a nucleoprotein fraction from spleen which is essential for *in vitro* proliferation of chick muscle tissue cells, and Harris(10) has demonstrated a requirement of chick muscle cells for a dialyzable factor.

However, even if the defects in cultures of dystrophic chick muscle cells could be controlled it would not mean that human dystrophic muscle grown *in vitro* would respond to the same factors. From our data it is apparent that human muscle differs from chick muscle both in respect to its growth pattern under optimal growth conditions and in its growth factor requirement. Human muscle loses protein during the first days of explantation while chick muscle maintains its protein content during this explantation period. Also, human muscle can grow *in vitro* without embryo extract but requires addition of a mixture of synthetic growth factors. Chick muscle requires embryo extract and a synthetic mixture of growth factors cannot be substituted for this.

Summary. 1) The changes in protein nitrogen and DNA contents of normal chick wing muscle tissue and of tissue from chick with muscular dystrophy were compared after culture in roller tubes. 2) No significant differences in the 2 parameters were found for normal and dystrophic tissue when grown in a medium containing 5% embryo extract. 3) When grown in 2.5% embryo extract the cultures from donors of an age of 2 weeks and older showed a statistically significant difference. Protein nitrogen of the cultures

from dystrophic muscle decreased during the 7-14 day culture period, whereas no comparable decrease was observed in cultures of normal tisue. Changes in the DNA content did not indicate definite differences. 4) Differences between several parameters determined in normal and dystrophic human muscle tissue cultures were found to be highly inconsistent or of no apparent relevance for the pathological state of the tissue.

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Received July 26, 1960. P.S.E.B.M., 1960, v105.

Changes in Liver Tyrosine-α-Ketoglutarate Transaminase Activity During Growth of Walker Carcinosarcoma 256. (26060)

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The tyrosine oxidizing system of liver is a series of enzymes converting tyrosine to fumarate and acetoacetate in the presence of α-ketoglutarate(1). A decreased tyrosine oxidation was observed in liver of rats fed a low protein diet(2). More recently, evidence has been presented suggesting that the lower level of tyrosine oxidation resulted from a decrease in activity of the initial enzyme in the tyrosine-oxidizing-system, tyrosine-α-ketoglutarate transaminase(3).

In addition to low protein diet, a decrease in liver tyrosine-α-ketoglutarate transaminase activity has been reported in rats subjected to tourniquet injury(4). An increase in activity of the enzyme has been reported following injection of hydrocortisone(5). An adaptive increase in tyrosine transaminase activity followed tyrosine injection. However, tyrosine was an effective stimulus only if the adrenal glands or hydrocortisone also were present(5). This discovery suggested that adrenal steroids played a major role in regulation of activity of this enzyme.

A number of studies have suggested an altered activity of the adrenal gland in tumor-bearing animals(6). Also, tumor-bearing animals generally exhibit a loss of appetite, particularly during late stages of tumor growth. It was therefore of interest to study the effect of tumor growth on liver tyrosine-α-ketoglutarate transaminase activity.

Methods. Female Holtzman rats weighing 160-200 g were used in these experiments. The Walker carcinosarcoma 256 was transplanted by injecting a cell suspension of tumor into both *rectus femoris* muscles according to the method of Talalay *et al.*(7). All animals were fed a diet of Rockland rat chow and water *ad libitum.*

Livers to be used for transaminase determination were excised and chilled immediately in cracked ice. After chilling, livers were perfused with cold isotonic saline and homogenized in a Potter-Elvehjem homogenizer with sufficient 0.15 M potassium chloride to give a 10% homogenate. The homogenates were centrifuged at 15,000 × g for 20 minutes at 0°C. Tyrosine-α-ketoglutarate transaminase activity was determined on the supernatant according to the method of Lin and Knox(5). Enzyme activity was expressed as μmoles of *p*-hydroxyphenylpyruvate formed per g dry weight of liver per hour.

To facilitate comparison of enzyme activity with tumor growth, animals were divided into 3 groups based on tumor weight as related to body weight. Tumors comprising less than 10% of body weight were considered "small," those 10-25% of body weight "medium," and those greater than 25% of body weight were designated "large."

Partial hepatectomies were performed according to the procedure of Higgins and Anderson(8). After 7 days animals were sacrificed and tyrosine-α-ketoglutarate transaminase activity determined on the regenerating liver.

Results. Data illustrating effect of growth of the Walker tumor on liver tyrosine-α-ketoglutarate transaminase activity are presented in Table I. No significant difference in enzyme activity was apparent in animals with tumors less than 10% of total body weight. A moderate increase in enzyme activity was noted in livers of rats with tumors 10-25% of body weight, while a 2-fold increase in transaminase activity was observed in livers from rats with tumors greater than 25% of total body weight. Thus, the tumor exerted little effect on liver tyrosine transaminase activity until the late stages of tumor growth.

An increase in glutamate-oxalacetate transaminase activity in regenerating liver 5-10 days following hepatectomy has been reported (9). It was of interest to determine if such an increase occurred with tyrosine transaminase since an increased mitotic activity is known to occur in livers of tumor-bearing animals(6). From data presented in Table II it was apparent that no change in tyrosine transaminase activity occurred in regenerating liver. Therefore, it seemed unlikely that the increase in enzyme activity observed in late stages of tumor growth was the result of an increase in mitotic activity.

TABLE I. Effect of Growth of Walker Tumor on Liver Tyrosine-α-Ketoglutarate Transaminase Activity.

Group	No. of animals	Enzyme activity*	P value†
Normal	16	134 ± 7‡	
Tumor§—Small	9	125 ± 9	>.200
Medium	8	188 ± 18	<.005
Large	13	269 ± 31	<.001

* Expressed as μmoles of p-hydroxyphenylpyruvate formed/g dry liver/hr.
† Compared with normals.
‡ Mean ± stand. error.
§ Small tumor, <10% body wt; medium tumor, 10-25% body wt; large tumor, >25% body wt.

TABLE II. Tyrosine-α-Ketoglutarate Transaminase Activity in Regenerating Rat Liver.

Treatment	No. of animals	Enzyme activity
Untreated	7	145 ± 8*
Sham	5	144 ± 12
Hepatectomy†	8	156 ± 11

* Mean ± stand. error.
† Hepatectomies were performed 7 days prior to transaminase determination.

Discussion. An elevated free tyrosine concentration in livers of tumor-bearing animals could account for the increased tyrosine transaminase activity. However, this possibility appeared to be eliminated since a lower than normal concentration of free tyrosine has been reported in livers of animals bearing the Walker tumor(10).

A 10-fold increase in liver tyrosine-α-ketoglutarate transaminase activity has been reported following injection of hydrocortisone (5). Unfortunately, little reliable information is available concerning functional status of the adrenal gland in tumor-bearing rats. However, Nadel and Burstein(11) have reported a 3-fold increase in urinary corticosteroids in late stages of tumor growth in guinea pigs. These authors suggested that the general stress associated with neoplastic diseases resulted in an increased adrenocortical activity. On the basis of available evidence, it appears that the increase in liver tyrosine-α-ketoglutarate transaminase activity in latter stages of tumor growth may be related to an increased adrenal steroid production.

Summary. Liver tyrosine-α-ketoglutarate transaminase activity was not affected by Walker carcinosarcoma 256 in the early stages of tumor growth. During latter stages of tumor growth a 2-fold increase in liver tyrosine transaminase activity was noted. No change was observed in tyrosine transaminase activity in regenerating liver; therefore, it

seemed unlikely that increased transaminase activity in the late stages of tumor growth resulted from an enhanced liver mitotic activity.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Selective Depression of Sympathetic Transmission by Intravenous Administration of Iproniazid and Harmine.* (26061)

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Gertner(1) demonstrated that when the superior cervical ganglion of the cat was perfused with monoamine oxidase (MAO) inhibitors, transmission through the ganglion was blocked. With low concentration of the inhibitors, the depression of transmission developed gradually, unlike the immediate blocking effects of hexamethonium. The present study was designed to compare the effects of intravenous administration of 2 MAO inhibitors, iproniazid and harmine, on transmission through sympathetic and parasympathetic ganglia of the dog and cat.

Methods and materials. (a) Dog Experiments: Mongrel dogs of both sexes were anesthetised with a mixture of pentobarbital, 15 mg/kg and barbital 225 mg/kg administered intravenously. Vagus nerves were cut bilaterally in the neck and mechanical respiration was provided. The chest was opened transversely, 2nd and 3rd ribs were removed, and a Walton strain gauge arch was sutured to the right ventricle. Heart contractile force and carotid arterial pressure were recorded with a Grass Polygraph as previously described(2). The stellate ganglion and sympathetic trunk were exposed on one side. The sympathetic trunk was crushed and ligated at the level of the 4th thoracic ganglion and a shielded 27 gauge silver wire electrode was placed on a presynaptic sympathetic nerve between 2nd and 3rd thoracic ganglia. A similar electrode was placed on a suitable postsynaptic sympathetic nerve. Pre- and post-synaptic nerves were stimulated by means of a Grass Model S-4G stimulator for 10 sec. periods at a frequency of 17 cycles/sec. with monophasic pulses of 0.5 sec. duration. Maximum increments in heart rate to increasing voltage was ascertained for pre- and post-synaptic nerves and the nerves were stimulated alternately with such voltage (ranging from 3-15 volts) during the course of experiment. The vagus was stimulated at 5-10 minute intervals with a fixed voltage which had previously been found to produce marked bradycardia. Anatomical dissection in the dog is described by Mizeres(3) and the technic for stimulation is essentially that de-

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* The authors wish to thank Mr. John Dolan for technical assistance. We are also indebted to Hoffmann-LaRoche, Inc. for supplies of iproniazid and isoniazid; to Charles Pfizer & Co., Inc. for harmine; and to Ciba Pharmaceutical Products, Inc. for Dial-Urethane.

† Submitted in partial fulfillment by F. M. DaCosta for M.S. degree from Howard Univ.

TABLE I. Effects of Iproniazid and Harmine on Transmission through Stellate Ganglion of Dog in 8 Experiments.

		Before inhibitor			After inhibitor			
Inhibitor	Dose, mg/kg	H.R.,* beats/min.	% increase Pre.	% increase Post.	H.R., beats/min.	% increase Pre.	% increase Post.	% inhibition† Pre.
Iproniazid	100	125	44	72	110	18	64	59
”	150	160	44	50	145	18	50	59
”	150	160	59	68	173	11	54	82
”	175	173	45	60	155	10	67	78
Harmine	5	167	49	49	120	25	63	49
”	5	130	66	62	70	21	144	68
”	10	175	37	57	130	0	69	100
”	15	115	130	118	105	14	95	89

* Heart rate. % increase in heart rate after preganglionic stimulation and after post ganglionic stimulation.

† $100 - \frac{\text{\% increase pre. after inhibitor}}{\text{\% increase pre. before inhibitor}}$.

scribed by Perry and Wilson for the cat(4). (b) Cat Experiments: Cats of both sexes were anesthetized with Dial-Urethane in a dose of 0.7 cc/kg administered intraperitoneally. Each cc contained 0.1 g diallyl barbituric acid, 0.4 g of urethane, and 0.4 g of monoethyl urea in aqueous solution. Pre- and postsynaptic sympathetic nerves of the superior cervical ganglion were separated from the vago-sympathetic chain, using care to leave intact the blood supply to ganglion and nerves. The ipsilateral nictitating membrane was connected to a Grass PR-4 strain gauge unit and isometric contractions of the membrane were amplified and recorded on a Grass Polygraph. Recordings of carotid artery pressure and electrical stimulations of the nerves were the same as described above. (c) Materials. Iproniazid phosphate (Marsilid, Hoffmann-LaRoche) and harmine, calculated as their bases, were administered intravenously in doses shown in Tables I and II. In the dog experiments, both agents were administered intermittently over a 15 minute period; in the cats, the drugs were given over a period of 30 to 120 minutes, to minimize their effects on the cardiovascular system.

Results. The actions of iproniazid and harmine on ganglionic transmission in both types of experiments were calculated as percent inhibition(4).

(a) Dog Experiments: The effects of iproniazid and harmine on transmission through the dog stellate ganglion are shown in Table I. As noted, both agents produced marked depression of the tachycardia resulting from preganglionic stimulation without decreasing the response to postganglionic stimulation.

The bradycardia produced by vagal stimu-

TABLE II. Effects of Iproniazid and Harmine on Transmission through Superior Cervical Ganglion of the Cat in 6 Experiments.

		Before inhibitor		After inhibitor		% inhibition
Inhibitor	Dose, mg/kg	N.M.–Pre.*	N.M.–Post.†	N.M.–Pre.	N.M.–Post.	Pre.‡
		mm				
Iproniazid	50	20	30	9	27	55
”	~~50~~	20	36	4	32	80
”	100	10	10	0	10	100
Harmine	12	31	31	23	31	26
”	20	29	23	16	22	45
”	25	26	28	7	25	73

* Height of recording of nictitating membrane contraction after preganglionic stimulation.
† *Idem* postganglionic ” .

‡ $100 - \frac{\text{N.M. pre. after inhibitor}}{\text{N.M. pre. before inhibitor}}$.

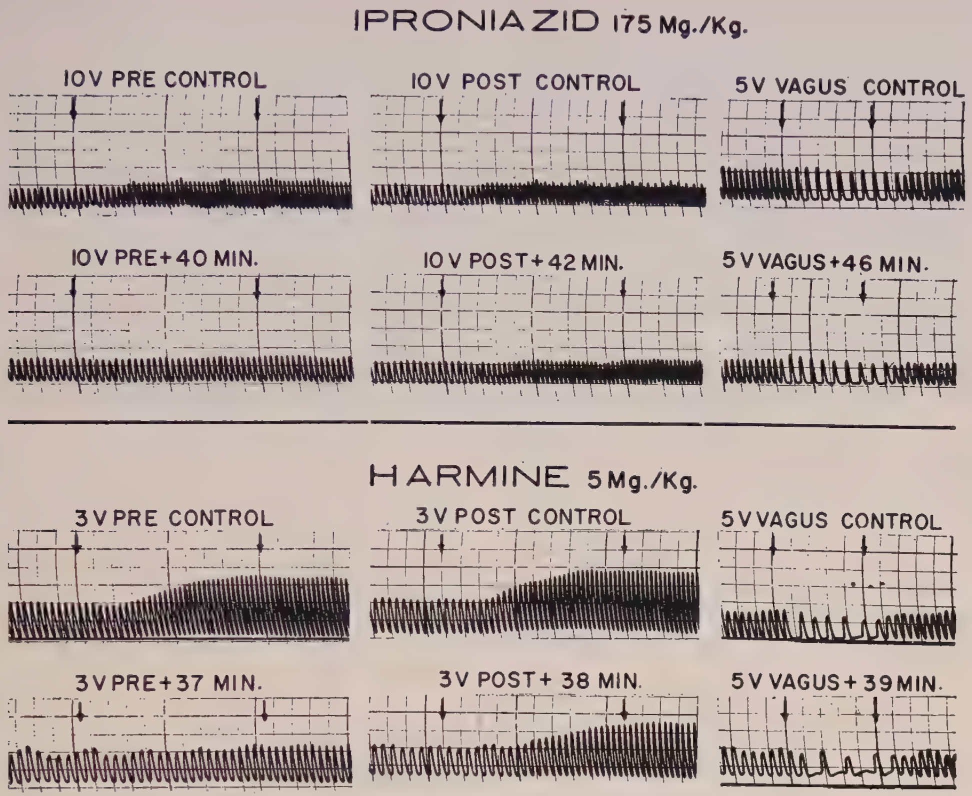

FIG. 1 (upper tracings). Effects of iproniazid, 175 mg/kg, intrav., on increases in heart rate and contractile force produced by maximal pre- and postganglionic stimulation of the stellate ganglion and on the bradycardia produced by stimulation of the vagus in an open-chest, anesthetized dog. The sympathetic nerves were stimulated for 10 sec. (indicated by the arrows), with 10 volts at a frequency of 17 cycles/sec. Vagus was stimulated for 5 sec. with 5 volts. Upper panels were obtained before and lower panels at designated time after completion of drug administration. (Lower tracings) Effects of harmine, 5 mg/kg, in a similar experiment. Conditions were the same except that maximal effects on heart rate and contractile force were obtained by stimulating pre- and postganglionic nerves with 3 volts.

lation was not affected by either agent and is therefore, not included in the table. In 2 additional experiments, the effects of stimulating the vagus at varying frequencies with constant voltage was also unchanged following intravenous administration of harmine, 10 mg/kg, and iproniazid, 150 mg/kg. Hexamethonium, 2 mg/kg, administered later in these experiments, completely blocked the effects of vagal stimulation. Fig. 1 illustrates increments in heart rate and contractile force produced by preganglionic stimulation which were almost totally blocked by the inhibitors. Also shown are the relatively unaffected postganglionic and vagal responses.

The sympathetic block produced by harmine was usually more rapid in onset and of shorter duration than that occurring with iproniazid. In 3 of the 4 harmine experiments, preganglionic responses had returned to between 60% and 90% of control values during the 120 minute period of observation. Preganglionic responses returned to only 20% of control values during a comparable observation period after administration of iproniazid.

Both iproniazid and harmine exerted marked cardiovascular actions and harmine also caused transitory convulsive movements in the large doses used, as previously described(2,5). In all experiments, harmine produced marked bradycardia which occurred immediately and was of relatively short duration. This effect preceded and was of shorter

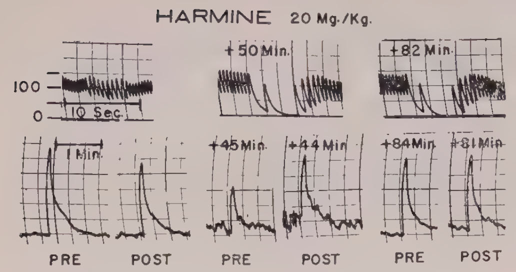

FIG. 2. Effects of harmine, 20 mg/kg, in an anesthetized cat. Upper tracings illustrate changes in arterial blood pressure (scale to left of tracing is mm Hg) and heart rate resulting from stimulation of the vagus nerve with 10 volts for 5 sec. at a frequency of 17 cycles/sec. Lower tracings are recordings of contractions of the nictitating membrane to stimulation of pre- and postganglionic fibers of the superior cervical ganglion with 10 volts for 10 sec. at a frequency of 17 cycles/sec. The first panels are before and the others are at designated times after completion of drug administration.

duration than the sympathetic block. Administration of iproniazid resulted in an immediate drop in mean blood pressure from levels of 100 to 140 mm Hg to values of 50 to 60 mm Hg without affecting heart rate or contractile force. This effect also preceded onset of significant sympathetic block, but persisted for duration of experiment.

As noted in Table I, 100% inhibition of sympathetic ganglionic transmission was observed in only one experiment. Attempts were made in several additional experiments to produce total block of sympathetic transmission by giving even larger doses of the inhibitors, but hypotension produced by iproniazid and convulsions and bradycardia caused by harmine usually became more pronounced and prevented evaluation of the ganglionic effects. In one experiment, however, an additional increment of 65 mg/kg of iproniazid was injected in divided doses during a 60 minute period and the 59% inhibition produced by 100 mg/kg of iproniazid was increased to 100%, without further hypotension. This experiment demonstrated that the hypotensive effect can be dissociated from the ganglionic action produced by iproniazid.

To evaluate the effects of a hydrazine which is not an MAO inhibitor, 150 mg/kg of isonicotinic acid hydrazide (INH) was administered intravenously in one experiment. There was no change in the effects of pre- and postganglionic stimulation during a 120 minute period of observation. Typical sympathetic block, however, resulted from the later administration of 150 mg/kg of iproniazid. Gertner(1) had previously demonstrated that INH did not inhibit transmission through the isolated superior cervical ganglion of the cat.

(b) Cat Experiments: Depression of transmission through the superior cervical ganglion was observed in 3 experiments with iproniazid and 3 with harmine (Table II). Bradycardia produced by vagal stimulation was not inhibited by either agent. Fig. 2 illustrates the pronounced depression of amplitude of contraction of the nictitating membrane to preganglionic stimulation and little change in amplitude to postganglionic stimulation following administration of harmine. The bradycardia produced by vagal stimulation was not inhibited, and in fact, appeared to be potentiated. A similar increase in vagal effect was noted in one other harmine experiment, but was not seen with iproniazid. Cardiovascular effects of the inhibitors were similar to those observed in the dog except that hypotensive effects of iproniazid were transient.

Discussion. Inhibition of transmission through sympathetic ganglia produced by intravenous administration of harmine and iproniazid in these experiments have confirmed the results obtained by Gertner(1) with the isolated superior cervical ganglion of the cat. The present results have additionally demonstrated that these agents exert a selective inhibitory action on the sympathetic synapse without depressing parasympathetic ganglia. More typical ganglionic blocking agents, such as hexamethonium, also inhibit parasympathetic ganglia, presumably by interfering with the action of acetylcholine (6). It is possible, therefore, that the depression of sympathetic transmission occurring with iproniazid and harmine results from a different mechanism of action. As iproniazid and harmine are otherwise similar only in ability to inhibit MAO, it is tempting to relate effects at the sympathetic ganglion to accumulation of amines metabolized by MAO. Administration of epinephrine and norepinephrine, for example, results in depression of ganglionic transmission(7).

One may only speculate that the selective sympathetic inhibition produced by large intravenous doses of iproniazid and harmine in the dog and cat is the mechanism responsible for postural hypotension in man after administration of MAO inhibitors. It is interesting, however, that the predominant autonomic effect of these agents in man appears to be sympathetic depression without parasympathetic block(8).

Summary. Large doses of iproniazid and harmine administered intravenously to the anesthetised cat and dog inhibit transmission through sympathetic ganglia without depressing the function of parasympathetic ganglia.

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Received April 12, 1960. P.S.E.B.M., 1960, v105.

Selenium and Torula Yeast in Production of Exudative Diathesis in Chicks.* (26062)

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Factor 3 and selenium have been shown to prevent exudative diathesis in chicks fed a Vit. E free Torula yeast diet(1,2). This, however, does not indicate that selenium is essential in nutrition of chicks when adequate Vit. E is present in the diet. Schwarz *et al.* (3) have suggested that Vit. E and selenium might participate in alternate pathways in metabolism in spite of marked structural differences. Participation of selenium in certain metabolic reactions has been reported by various workers(4,5,6). This element has also been reported to promote growth in chicks and rats(1,4,5). Dried brewers yeast, which is free of Vit. E but is a potent source of Factor 3(7), will replace Vit. E in preventing exudative diathesis in chicks (8). A growth response attributed to Vit. E was observed in chicks fed a high fat diet(9) but recent work shows that Vit. E was not needed by chicks fed a diet low in fat(10,11). Bieri *et al.*(12) reported that addition of Torula yeast to an otherwise purified chick diet resulted in production of exudative diathesis in 16-18 days, and provided evidence for existence of factors of a fatty and mineral nature in Torula yeast which stimulated production of exudative diathesis in chicks fed a Vit. E free diet. The experiments reported here were designed to study the effects of selenium, Vit. E and dried brewers yeast on growth of chicks fed a purified diet containing soybean protein, in an attempt to provide additional information on the role of such ingredients in nutrition of chicks. Investigations were also made of the relation between Torula yeast and production of exudative diathesis in the chick.

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* These studies supported in part by grant-in-aid from Welch Foundation, Houston, Texas. The following products were supplied through the courtesy of indicated companies: choline chloride by Hoffman-Taff, Penicillin and other B-vit. by Merck and Co., D-alpha-tocopheryl acetate by Distillation Products Industries, condensed fish solubles by Philip R. Park, aureomycin by Am. Cyanamid Co., stabilized vit. A by Stabilized Vitamins, vit. D_3 by Charles Bowman and Co., menadione sodium bisulfite by Heterochemical Corp., butylated hydroxytoluene by Koppers Co., and Torula yeast by Lake States Yeast Corp.

TABLE I. Composition of the Basal Diet.

	%		%
Glucose monohydrate	53.84	Wood pulp	3
Drackett C-1 assay protein	32	Vitamins*	1.32
Soybean oil	3	Minerals†	6.84

* The vitamin premix supplied the following/kg of diet: Thiamine hydrochloride, 10 mg; riboflavin, 10 mg; D-calcium pantothenate, 20 mg; pyridoxine hydrochloride, 6 mg; para-aminobenzoic acid, 20 mg; inositol, 500 mg; biotin, 0.2 mg; vit. A, 10,000 I.U.; vit. D_3, 400 I.C.U.; niacin, 75 mg; folic acid, 4 mg; BHT, 500 mg; methionine, 7,500 mg; glycine, 3,500 mg; choline chloride, 1,500 mg; menadione sodium bisulfite, 0.5 mg; vit. B_{12}, 0.02 mg.

† The mineral supplement provided the following /kg of diet: $CaCO_3$, 10 g; $CaHPO_4 \cdot 2H_2O$, 38.89 g; NaCl, 6 g; $MnSO_4 \cdot H_2O$, 0.37 g; $MgSO_4 \cdot 7H_2O$, 5.27 g; $FeSO_4 \cdot 7H_2O$, 0.1478 g; $CuSO_4$, 0.011 g; $CoCl_2 \cdot 6H_2O$, 0.0016 g; KCl, 7.63 g; $Na_2MoO_4 \cdot 2H_2O$, 0.00076 g; KI, 0.007 g.

The above diet contains: 25.92% protein, 2.38 calories productive energy/g.

Methods. In the first study day-old New Hampshire chicks were distributed at random in groups of 20 in electrically heated battery brooders with raised wire floors. The birds were fed a purified soybean protein diet (Table I) supplemented with selenium, Vit. E or dried brewers yeast (Table II). In the second study day-old White Plymouth Rock chicks, 20 birds per group, were fed the purified diet shown in Table I, with soybean oil being replaced by Vit. E-free molecularly distilled lard.† Dietary supplements tested in this experiment included selenium (as sodium selenate), Vit. E, dried brewers yeast, Torula yeast, salts of sodium, potassium, magnesium and arsenic, and various combinations of these supplements. Feed and water were supplied *ad libitum* and body weights were taken at weekly intervals throughout the studies. Daily observations were made for appearance of symptoms of exudative diathesis. When sufficient birds in the basal group showed symptoms of exudative diathesis, 5 representative birds were removed from each group for biochemical studies. Blood was collected from the chicks by heart puncture and allowed to clot for one hour at room temperature, and was subsequently centrifuged at 3000 rpm for 5 minutes to obtain the serum. Total serum protein was determined by the method of Kingsley(13) and albumin/globulin ratio (A/G ratio) in the serum was determined using the paper electrophoresis technic.‡ Hemoglobin concentration in blood obtained by wing vein puncture was determined by the alkali hematin method.

Results. Addition of selenium, Vit. E or dried brewers yeast to the basal diet resulted in a marked growth improvement. Dietary supplements of 40 mg of *d*-α-tocopheryl acetate per kg of diet, 0.1 ppm selenium, and 10% dried brewers yeast resulted in growth responses of 9.6 to 11.0% over the birds fed the basal diet. Addition of 0.05 ppm selenium to basal diet resulted in a growth response of about 14% compared to the unsupplemented group. Total serum protein level of the chick, however, did not show any differences attributable to the supplements, indicating that either the selenium or Vit. E in the basal diet was adequate to maintain normal protein level in the serum (Table II). Similarly, no symptoms of exudative diathesis were observed during this experiment (Table II).

The basal diet (Table I) contained soybean oil, which supplied an estimated 4 mg of α-tocopherol per kg of diet. When soybean oil was replaced with Vit. E free lard, the basal diet had no appreciable Vit. E content but might have contained selenium. The birds

TABLE II. Effect of Selenium on Growth of Chicks Fed a Purified Soybean Protein Diet (20 Chicks/Treatment).

Supplement to basal diet	Wt in g, 21 days	Incidence of exudative diathesis, %	Total serum protein, g %, 25th day
None	365 (10.3)§	0	4.3
Vit. E*	406 (11.1)	0	4.1
D.B.Y.†	401 (10.3)	0	4.4
Selenium‡			
.1 ppm	400 (11.8)	0	4.4
.05 "	415 (11.2)	0	4.2

* 40 mg *d*-α-tocopheryl acetate/kg of diet.

† Dried brewers yeast, 10% of diet.

‡ As sodium selenate.

§ Values in parentheses are stand. errors of group means.

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† Obtained from Distillation Products Industries, Rochester, N. Y.

‡ Spinco Model R paper electrophoresis apparatus and Spinco Model RB Analytrol, Spinco Division, Beckman Instruments, Belmont, Calif.

TABLE III. Effects of Different Supplements on Growth and Incidence of Exudative Diathesis in Chicks Fed a Purified Soybean Protein Diet for 35 Days (20 Chicks/Treatment).

Supplement to basal diet	Avg wt, g	Incidence exudative diathesis, %	Day of appearance of symptom	Total protein, g/100 ml	A/G ratio	Hemoglobin, g/100 ml
None	503 (12.9)[§]	5	30	5.84	.60	7.04
Vit. E[*]	564 (14.3)	0		6.90	.93	8.90
D.B.Y.[†]	566 (14.1)	0				
Selenium[‡]	575 (15.0)	0				
Vit. E + D.B.Y.	565 (13.7)	0				
D.B.Y. + selenium	568 (13.5)	0				
Selenium + vit. E	572 (14.0)	0				
Vit. E, D.B.Y. + selenium	571 (13.8)	0				
1% K (KCl) + .07% Mg ($MgSO_4 \cdot 7H_2O$)		5	30			
.7% sodium ($Na_2SO_4 \cdot 7H_2O$)		6	29			
.5% ppm of arsenic		4	31			
20% Torula yeast		20	18			
30% *idem*		19	17			

[*] 40 mg *d*-α-tocopheryl acetate/kg of diet.
[†] Dried brewers yeast, 10% of diet.
[‡] .05 ppm of diet as sodium selenate.
[§] Values in parentheses are stand. errors of group means.

fed this diet developed symptoms of exudative diathesis beginning on 30th day of study and by the 35th day, 25% of the birds showed symptoms of the condition (Table III). Affected birds had a mild edema and greenish exudates with subcutaneous hemorrhage in the abdomen, but were otherwise normal. Each of the supplements, selenium, Vit. E and dried brewers yeast, prevented the symptoms of exudative diathesis. It seems evident that any selenium present in the soybean protein was unavailable or available only in traces. It should be noted that Torula yeast contains 1.6 ppm of selenium which is unavailable (14). Total serum proteins and A/G ratios, and hemoglobin contents of the blood of the affected birds were reduced by 16%, 35% and 21% respectively when compared to those of the birds receiving Vit. E (Table III). Reduction in total protein and A/G ratio of the serum was not as marked as has previously been noted in affected birds fed a Torula yeast diet(8). This may be related to the mild symptoms of exudative diathesis observed in chicks fed the soybean protein basal diet. The supplements, singly or in combination, exerted similar growth-promoting effects at the end of the fifth week (Table III) and each of the supplements prevented exudative diathesis, thus indicating that dried brewers yeast or selenium can replace Vit. E for chicks. Furthermore selenium could account for the beneficial effect of dried brewers yeast on growth of chicks and in prevention of exudative diathesis, since dried brewers yeast has been reported to be a potent source of selenium but to contain no Vit. E(7).

It has been reported that the time needed to produce exudative diathesis in chicks fed a purified soybean protein diet was 30 days, compared to only 17 days when a Torula yeast diet was fed to the chicks(8). The appearance of the deficiency condition was also much less severe in the former case. In the present experiment the use of a diet which contained 20% Torula yeast, purified soybean protein and Vit. E-free stripped lard decreased time of appearance of exudative diathesis from 30 days to only 18 days, while with a similar diet containing 30% Torula yeast the time was reduced to only 17 days (Table III).

Since Torula yeast is rich in potassium and magnesium(15) these minerals were added in combination at levels of 1% and 0.07% respectively to the soybean protein purified diet. Arsenic has been reported to counteract selenium toxicity in chicks(16) and an excess of sodium will cause potassium excretion and may thus cause an imbalance of the osmotic mechanism. Since exudative diathesis has been shown to be accentuated by additional salts in the diet(17) 2 groups were fed the soybean-protein diet supplemented with 0.7% sodium (from Na_2SO_4) or 0.5 ppm of arsenic (from sodium arsenate). The symptoms of exudative diathesis, however, were neither hastened nor accentuated by these supplements (Table III).

Summary. Vit. E, dried brewers yeast, and 0.05 ppm selenium promoted growth and prevented symptoms of exudative diathesis in chicks fed a purified soybean protein diet. Any of the supplements completely replaced the others and maintained normal values for serum protein, A/G ratio and blood hemoglobin. Chicks fed Vit. E-free diet, containing purified soybean protein as source of protein, developed symptoms of exudative diathesis in 30 days, manifested by mild edema and exudation with subcutaneous hemorrhage in the abdomen. The affected birds had a reduced serum protein level, A/G ratio, and blood hemoglobin level. Addition of 0.7% sodium, 0.5 ppm arsenic or a mixture of 1% potassium and 0.07% magnesium, to above Vit. E-free, soybean-protein diet failed to reduce the time needed for development of exudative diathesis. Addition of Torula yeast (20 or 30% of the diet) to Vit. E-free soybean protein diet hastened and increased incidence of exudative diathesis in chicks fed this diet. Thus it appears that some component in Torula yeast affects onset of exudative diathesis.

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Received April 19, 1960. P.S.E.B.M., 1960, v105.

Comparison of Various Sources and Levels of Vitamin K Activity Using Chicks with Cecal Coccidiosis.*† (26063)

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(Introduced by R. L. Shirley)
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Harms and Tugwell(1) reported that mortality of chicks was significantly increased by addition of dicumarol to the diet of chicks suffering from cecal coccidiosis. This indicated that "normal" blood clotting time was

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* *Florida Agri. Exp. Sta. Journal* Series, No. 1091.

† The authors are indebted to Abbott Laboratories, North Chicago, for menadione sodium bisulfite and menadione; Merck Sharp & Dohme, Rahway, N. J., for vit. K_1; and Chas. Pfizer & Co., Terre Haute, Ind., for vit. A and B-vitamins.

TABLE I. Composition of Basal Diet.

	%		%
Ground yellow corn	65.4	Ground limestone	.7
Soybean oil meal (50% protein)	31.0	Iodized salt	.4
		Micro-ingredients*	.7
Defluorinated phosphate (34% Ca & 17% P)	1.8		

* Supplies/lb of feed: 2268 I.U. vit. A, 10 μg vit. B_{12}, 340 I.C.U. vit. D_3, 2 mg riboflavin, 9 mg calcium pantothenate, 18 mg niacin, 261 mg choline chloride, 10 mg terramycin, 2 mg oleandomycin, 57 mg santoquin, 9 mg iron, 0.9 mg copper, 90 μg cobalt, 5 mg iodine, 45 μg zinc, 80 mg manganese sulphate, and 35 mg manganous oxide.

necessary to prevent excessive mortality due to internal bleeding during an outbreak of coccidiosis. Later reports(2,3) indicated that addition of menadione sodium bisulfite complex‡ to a Vit. K-low diet would significantly reduce mortality of chicks suffering from cecal coccidiosis. The purpose of this work was to determine the effect of feeding graded levels of MSBC to chicks when they were submitted to the stress of acute cecal coccidiosis, and to compare the relative value of various sources of Vit. K activity by their protection against mortality from coccidiosis.

Methods. Four experiments have been conducted with day-old Vantress X White Plymouth Rock chicks. In the first 2 experiments, diets were fed which contained graded levels of MSBC (0.0 to 4.56 g/ton of feed). In the third and fourth experiment diets containing various levels of MSBC, menadione and Vit. K_1 were fed. The composition of the Vit. K-low basal diet is shown in Table I. Six replications of 7 males and 7 females, and 5 replications of 6 males and 6 females were used in Exp. 1 and 2, respectively. Six replications of 5 males and 5 females per treatment were used in Exp. 3 and 4.

All chicks were reared in electrically heated batteries equipped with thermostatic temperature controls and raised wire floors. Experimental diets were fed *ad libitum,* from one day of age through the entire experimental period. Water was also kept before birds at all times. The water was changed every other day and the containers were cleaned and disinfected with a chlorine solution to minimize bacterial synthesis of Vit. K.

Birds were inoculated at 21 days of age with sporulated oocysts of *Eimeria tenella,* with each bird receiving 50,000 oocysts in Exp. 1, and 40,000 in Exp. 2, 3 and 4. One replicated group was not inoculated in Exp. 1 and 2, which served as a control to establish normal gain for the experimental period.

Individual body weight of chicks in Exp. 1 and 2 was obtained at 3 and 6 days post inoculation. Rate of gain during this 4-day period was used as a criterion of morbidity.

Results. Fig. 1 shows that as the level of dietary MSBC was increased from 0 to 1.14 g/ton of feed, mortality of chicks suffering from cecal coccidiosis was reduced linearly to approximately 40 to 50%.

Internal bleeding has been shown to be the primary cause of death from coccidiosis(1). Almquist(4) reported that dosage of Vit. K and prothrombin time has a linear relationship. The present data suggest that MSBC is effective by decreasing the internal bleeding caused by the disease.

Morbidity of chicks was reduced as level of MSBC was increased (Fig. 2). Growth rate of all inoculated birds was severely depressed

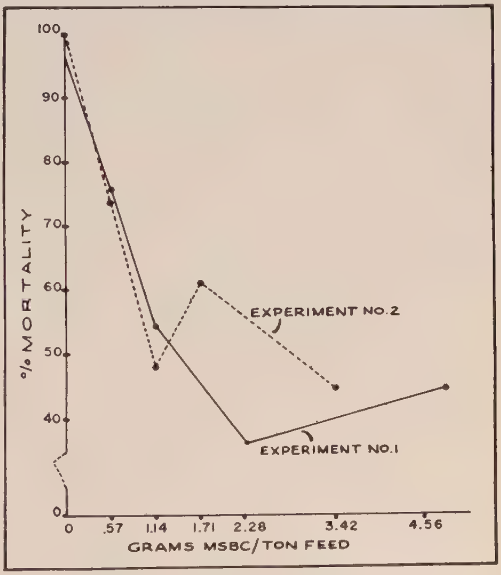

FIG. 1. Mortality of chicks receiving various levels of MSBC when suffering from cecal coccidiosis.

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‡ Menadione sodium bisulfite complex is defined to contain not less than 63% menadione sodium bisulfite U.S.P., hereafter referred to as MSBC.

as normal birds gained 65 and 50 g in Exp. 1 and 2, respectively. The relationship between morbidity and dietary level of MSBC also appears to be linear; however, differences were not as great as was reflected by mortality rates, indicating that mortality would be a better measurement of Vit. K activity from different sources.

Results from 2 experiments (Table II) based on mortality from coccidiosis indicate that MSBC on a weight basis is 6 to 7 times as effective as menadione. It also appears that Vit. K_1 may be 4 times as effective as MSBC. Comparative value of these sources of Vit. K are not in complete agreement with earlier reports(5,6,7). However, it should be pointed out that these workers were using sulfaquinoxaline, a Vit. K antagonist, which may not have indicated the chicks' true ability to utilize the source of Vit. K.

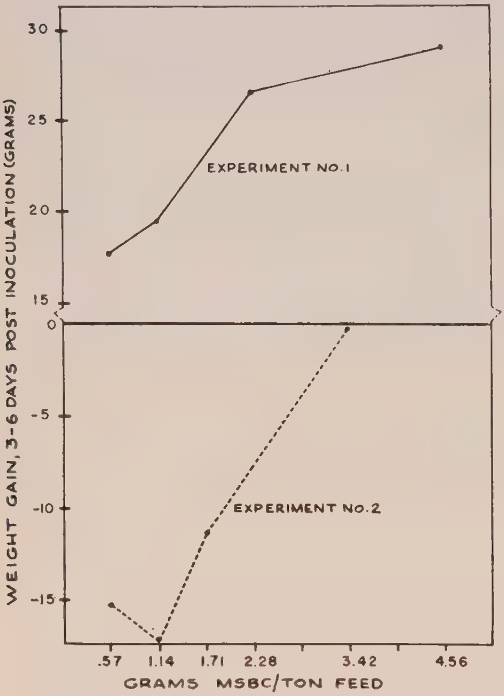

FIG. 2. Weight gain of chicks, 3-6 days post inoc., receiving various levels of MSBC when suffering from cecal coccidiosis.

TABLE II. Mortality of Chicks from Coccidiosis When Fed Various Levels and Sources of Vitamin K.

Vit. K source	g/ton	% mortality*
	0	98.1^{a}
MSBC	.36	83.3^{ab}
Menadione	2.07	77.3^{bc}
"	4.14	68.1^{cd}
"	6.21	64.6^{cde}
MSBC	.72	60.6^{def}
K_1	.68	57.8^{def}
MSBC	1.08	54.8^{def}
K_1	.23	52.5^{ef}
K_1	.46	46.3^{f}

* Means having different exponential letters are significantly different according to Duncan's Multiple Range Test (1955).

Summary. Data have been presented which indicate that increased dietary levels of MSBC result in a decreased mortality and morbidity of chicks suffering from cecal coccidiosis. Based on mortality from coccidiosis on a weight basis, MSBC is 6 to 7 times as effective as menadione. Vit. K_1 appears to be 4 times as effective as MSBC.

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Received May 19, 1960. P.S.E.B.M., 1960, v105.

Lymphatics in Intestinal Absorption of Vitamin B_{12} and Iron.* (26064)

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Intestinal malabsorption may be due to obstructed mesenteric lymphatics(1), and is often accompanied by disturbed B_{12} and iron absorption(2,3). For this reason, the role of the mesenteric lymphatics in transport of iron and Vit. B_{12} from the intestinal wall to the blood was studied in 2 dogs with cannulated thoracic ducts.

Materials and methods. Two beagles (weight 35 and 22.5 lb) were fasted for 12 hours. The left thoracic duct was isolated and cannulated at its entrance into the left jugular vein under ether anesthesia. The external jugular vein on the opposite side was identified, cannulated, and an external shunt established between the thoracic duct and this jugular vein. The shunt was in operation whenever lymph or blood samples were not drawn.

Both dogs were given 3.9 μc/4 μg of Co^{58}-B_{12} and 4 μc/1 μg of Fe^{59}-citrate by stomach tube. Blood and lymph samples were taken, and lymph flow rates measured at half hourly or hourly intervals during 8 hours. Radioactivity was measured in a Penco 100-channel pulse-height analyzer equipped with a 3 inch well-type scintillation detector. The 0.81 Mev Co^{58} and 1.10 Mev Fe^{59} peaks were employed. Counts were corrected for the contribution of Co^{58} summation radiation in the Fe^{59} peak and the Fe^{59} Compton radiation in the Co^{58} peak.

Total hourly radioactivities reaching the blood stream *via* lymph were approximated by assuming that the mean of 2 consecutive flow rates represented the true mean flow rate during that time. Total plasma volume was assumed to be 4.2% of total body weight (4).

The state (bound or free) of the radioactivity in the lymph samples was assayed by concentration dialysis against a 25% (w/v) solution of polyvinyl pyrrolidone. The dialysis method for determining free B_{12} binding capacity has been described(5).

Animals were sacrificed 10 hours and 7 days, respectively, after ingestion of isotopes. Excreta, intestinal contents, and intestines were collected, liquefied with HNO_3, concentrated by evaporation, and radioactivity was measured.

Results. At both times of sacrifice, most of the B_{12} was recovered in the intestine and excreta, whereas most of the iron had been absorbed (Table I).

In plasma, the time pattern was practically identical for both tracers, and there was little variation between the 2 animals. Serum peaks occurred 4-5 hours after ingestion for both B_{12} and iron (Fig. 1-2, Table II).

In lymph, both radio-iron and radio-B_{12} concentration-peaks came 2 to 3 hours after ingestion, *i.e.*, before the corresponding serum peaks. This was true for both animals (Table II, Fig. 3, 4).

Since more of the radio-iron was absorbed, Fe^{59} concentrations were higher in lymph and plasma than radio-B_{12} concentrations (Table II). However, while radio-iron concentration in lymph did not differ significantly from that in serum, radio-B_{12} concentration was 10 to 20 times higher in serum than in the lymph for both dogs. The total cumulative fraction of iron and B_{12} reaching the blood *via* the lymph was only about 1.3% of total iron dose given and 0.05% of total B_{12} dose given (Fig. 2, 4). Thus, most of the iron and B_{12} absorbed does not go *via* the lymph.

In those lymph samples where radio-iron concentration was low (0.06 mμg Fe^{59}/ml), no iron was dialyzable, but where it was high (0.2-0.3 mμg Fe^{59}/ml), 70% of the iron was dialyzable. Since the average unsaturated iron binding capacity in normal human serum

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* Research supported by U. S. Atomic Energy Comm. and in part by grant from Nat. Cancer Inst., N. I. H., U. S. P. H. S.

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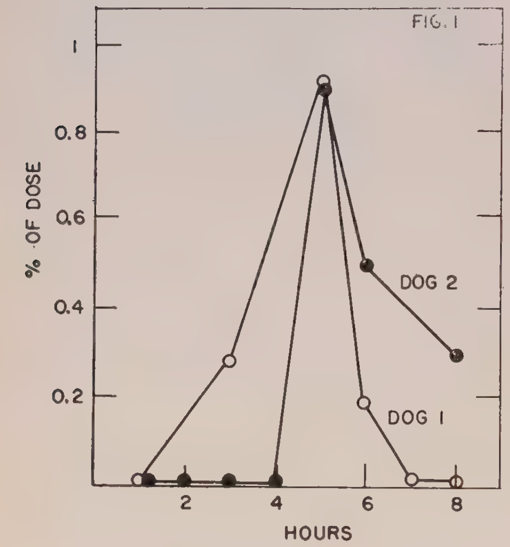
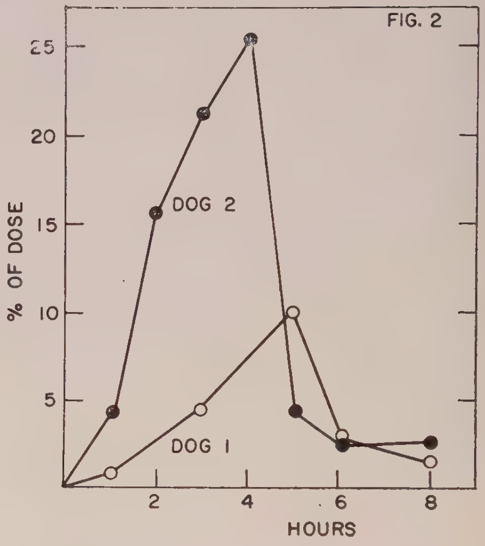
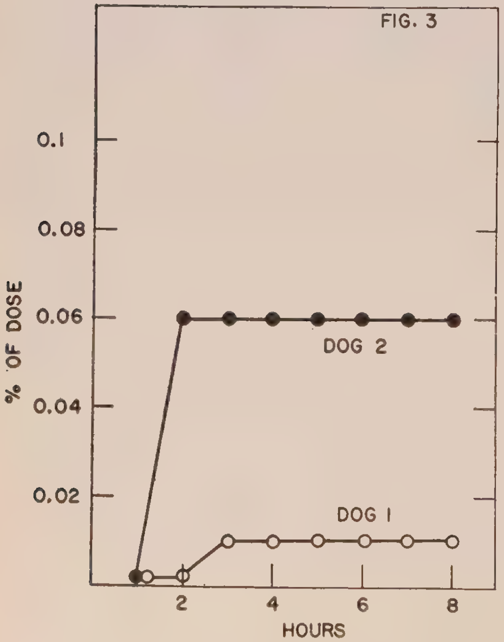
TABLE I. Radio-B_{12} and Radio-Iron Recovered in Excreta, Intestinal Contents and Intestines after Administration via Gastric Tube.

Dog	Times, admin./sacrifice	Amt B_{12} given, μg	Amt Fe given, μg	Amt B_{12} recovered μg	Amt B_{12} recovered % of dose	Amt Fe recovered μg	Amt Fe recovered % of dose
1	Nov. 17, 0800/Nov. 17, 1800	4	1	3.75	94	.33	33
2	Jan. 12, 0900/Jan. 19, 1200	4	1	1.76	44	.35	35







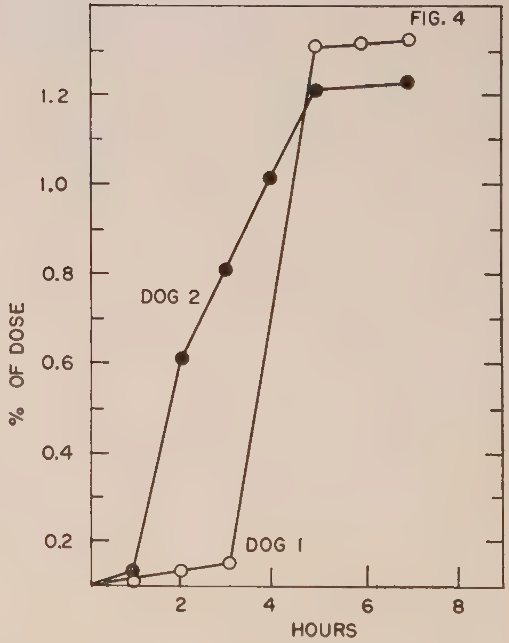


FIG. 1. B_{12} in total plasma—vol; % of dose.
FIG. 2. Fe in total plasma—vol; % of dose.
FIG. 3. Cumulative Co^{58}-B_{12} reaching blood via lymph; % of dose.
FIG. 4. Cumulative Fe^{59} reaching blood via lymph.

TABLE II. Concentrations of Co^{58}-B_{12} and Fe^{59} in Lymph and Plasma after Gastric Tube Administration.

		Peak concentration					
		Lymph		Plasma		Hr after ingestion	
	Dog	mμg/ml	% of given dose/ml	mμg/ml	% of given dose/ml	Lymph	Plasma
Fe^{59}	1	.34	34×10^{-3}	.46	46.4×10^{-3}	4	5
	2	.17	17×10^{-3}	.17	17.3×10^{-3}	2	4
Co^{58}-B_{12}	1	.003	$.07 \times 10^{-3}$	.07	1.7×10^{-3}	3	5
	2	.005	$.12 \times 10^{-3}$	.06	1.5×10^{-3}	2	5

is about 2 μg/ml(6) the iron binding capacity in dog lymph seems to be less than 1/1000 of what is found in human plasma.

The free B_{12} binding capacity in lymph, after adding 25 mμg/ml of Co^{60}-B_{12}, was 0.7 and 1.7 mμg/ml, respectively, which is comparable to that found in the plasma from 1 dog (3 mμg/ml) and to that in normal human plasma (6 mμg/ml) (Fig. 5).

Discussion. It has been demonstrated previously that iron transport takes place mainly *via* portal blood(7-10). This is confirmed by the present study, which indicates that Vit. B_{12} also is transferred directly from the intestinal wall to the portal blood without going *via* the lymph.

The time relationship between concentration of absorbed radio-iron in plasma and lymph has not previously been studied. The fact that both radio-iron and radio-B_{12} concentrations in lymph reached their peaks earlier than those in blood indicates that a small fraction of these substances is actually absorbed *via* the lymph while intestinal concentration is high. Later, most of the iron found in the lymph probably comes from the plasma(7,10). Since the concentration gradient between plasma and lymph is higher for radio-B_{12} than for radio-iron, and since it has been shown that plasma-radio-B_{12} escapes rapidly into the extravascular space(11,12) it is probable that much of the radio-B_{12} found in the lymph is also derived from plasma, at least after plasma concentration has reached its maximum.

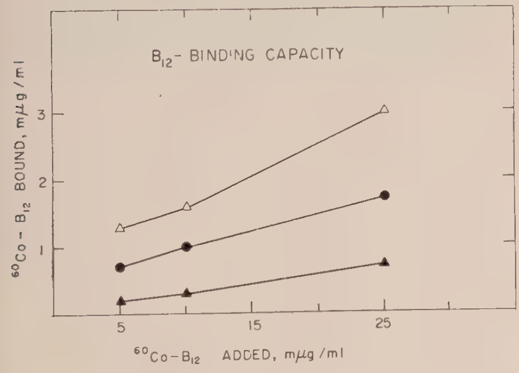


FIG. 5. Unsaturated B_{12}-binding capacity in dog lymph (filled symbols) and dog plasma (empty symbols).

There are indications that substances with low molecular weights such as fatty acids with less than 10 carbon atoms(13), amino acids (13-15), and hexoses(13), are mainly transferred directly from the intestine to the portal blood, whereas larger molecules, such as fatty acids with more than 10 carbon atoms(13,15) or native proteins(16,17), are transferred into the lymph. The possible malabsorption of B_{12} in steatorrhea due to blocked lymphatics may be secondary to the presence of fat in the intestinal lumen, in analogy with the findings in pancreatogenic steatorrhea (18).

There has been considerable discussion whether Vit. B_{12} is absorbed as a compound with a molecular weight only slightly higher than that of pure crystalline B_{12}(19,20) or whether it is accompanied by the entire intrinsic-factor-molecule (molecular weight about 70,000). Such a large complex would probably be transported *via* the lymphatic route. The present observations indicate, therefore, that most of the absorbed radio-B_{12} is absorbed as a considerably smaller molecule.

If this is true, then the degradation of intrinsic factor must take place in the intestinal wall, for it has been shown that the enzymes present in the intestinal lumen are not capable of splitting the intrinsic factor-B_{12}-complex(21). As a matter of fact, during preparation of the present paper, such an intracellular enzyme in the intestinal wall was described(22,23). Defects in this intracellular enzyme may explain the resistance to hog intrinsic factor in patients. It is also conceivable that the time lag before B_{12} appears in the blood (Fig. 1, dog 1)(24) is explained by this process in the intestinal wall.

Simultaneously with this work, experiments in rats by Taylor and French were published (25), which are confirmed by our results.

The emerging picture of B_{12}-absorption resembles that of iron absorption. During several hours after entering the intestinal wall, the major portion of an originally protein-bound molecule is retained in the intestinal wall, where it is separated from its original binder before being transferred into the portal blood. Meanwhile, a very small fraction, possibly still bound to the original binder is absorbed *via* the lymph.

Summary. Transport of radio-iron and radio-B_{12} from the intestinal wall to the blood was studied in 2 dogs with cannulated thoracic ducts. Most of both substances was directly transferred into the blood without going *via* the lymph. Since large molecules are generally absorbed *via* lymph, this indicates that the large B_{12}-intrinsic factor is split in the intestinal wall prior to transfer.

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Received May 31, 1960. P.S.E.B.M., 1960, v105.

Excretion of 5-Hydroxyindoleacetic Acid Following Bowel Ischemia in Dogs. (26065)

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Since the major part, perhaps 90-95%, of the body's serotonin (5-hydroxytryptamine) is located in the gastrointestinal mucosa(1), this study was designed to determine whether production of severe intestinal ischemia in dogs would influence urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA), the end product of serotonin metabolism.

Method. Fasting greyhound dogs of both sexes, weighing between 22 and 32 kg with an average weight of 25 kg, were used. Three experiments were performed utilizing 4 animals in each experiment. Following production of general anesthesia with intravenous pentobarbital sodium, an intravenous infusion was initiated which ran throughout the 6 hour test period. The infusion delivered approximately 7 to 8 cc of 5% dextrose in water/kg body weight/hr. The bladder of each animal was catheterized, the first sample of urine discarded, and urine collected hourly thereafter. The 5-HIAA content of each urine sample was determined by the method of Udenfriend *et al.*(2). In all animals, urine was collected for 2 hours before any procedures were performed. Then 2 test conditions and 2 controls were established as follows: 1) Control Group: Laparotomy only (3 animals). 2) Control Group: Laparotomy and ligation of terminal aorta producing ischemia of the hind quarters (3 animals). 3) Test Group: Laparotomy and ligation of the arterial supply to the mesenteric small bowel followed by strangulation of the mid 2 feet of small intestine by a cord (6 animals). Following institution of test or control conditions, urine specimens were collected for an additional 4 hours.

Results. Mean basal excretions of 5-HIAA are shown in Column 1 of Table I. Statistical evaluation utilizing an analysis of covariance reveals *f* value of 1.67 which for 3 and 8 degrees of freedom is not significant at the 0.05 level. This indicates that there was no initial difference between animals tested.

TABLE I. Mean Hourly Excretion, mg/Hr, of 5-Hydroxyindoleacetic Acid.

	Mean hourly amounts (mg) in 3 groups		
	Test control		Test
Basal excretion	Laparotomy	Aorta ligation	bowel ischemia
.55	.33		
1.10	.46		
.75	1.93		
1.45		.26	
.46		.24	
.36		.53	
.40			.11
.40			.16
1.20			.40
1.60			1.25
.41			.31
.73			.63

During the subsequent 4 hours' excretion of 5-HIAA, within-group variation was greater than between-group variation, consequently a probability value could not be determined (Table I). This indicates, however, that post operative differences were not significant. Using the preoperative values to predict what would be expected, statistical results revealed a *f* of 2.15 which suggests that some changes occurred, but were not great enough to be significant. Neither was there a significant difference between test and 2 control groups.

Conclusions and summary. Although it is not possible to determine the effect of ischemia on serotonin contents of bowel mucosa by this experiment, urinary excretion of 5-HIAA in large dogs does not change in the first 4 hours after production of severe bowel ischemia.

The authors are grateful to Lee Dahlbert for performance of 5-HIAA determinations, Dr. Jack Sandler for statistical analysis, and to Tom Lin-

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goya and Tom Kelly for technical assistance.

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Received June 6, 1960. P.S.E.B.M., 1960, v105.

Inhibition of Gastric Secretion in the Rat By Normal and Abnormal Human Gastric Juice.* (26066)

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Human gastric juice contains an unidentified substance which, when injected intravenously, inhibits gastric secretion in the dog and in the rat(1,2). Brunschwig and associates found that gastric juice collected from patients with gastric cancer had a greater inhibitory effect on canine gastric secretion than that collected from normal individuals (3). Previously we reported that intravenous injection of normal human gastric juice consistently suppressed gastric secretory activity of pylorus ligated rats(4). Because of the small total doses of gastric juice required to produce inhibition and the possibility of conducting many experiments in a short period of time, the pylorus ligated rat appeared to be a good preparation for assaying inhibitory activity of human gastric juice collected under various conditions. The present report, using pylorus ligated rats, is concerned with comparison of gastric inhibitory activity of normal human gastric juice with that of gastric juice from patients with duodenal ulcer and gastric cancer.

Materials and methods. Gastric juice was collected from hospital patients either free from gastrointestinal disease or with proven duodenal ulcer or gastric cancer. Because of the relative scarcity of gastric cancer patients, juice from these patients was processed individually and pooled later in lots of 3. Processing of gastric juice and methods of studying effects of gastric juice material on gastric secretory activity in rats have been described (4). Control rats were given intravenously 1 ml of normal saline; test rats received 6 mg/200 g of normal human gastric juice preparation, cancer juice preparation, or ulcer juice preparation in 1 ml of normal saline. Experiments were performed in series of 20 rats with 5 rats each of control and 3 test groups. The mean 4 hour output of HCl in milliequivalents of each test group was compared with that of control group. Initially 60 rats were studied in this fashion. Several months later the same experiments were repeated on 60 rats of a different colony and with gastric juice material from different pools.

Results. The data from the 2 series of experiments are outlined in Table I. In rats receiving 6 mg of normal human gastric juice preparation, there was 85% and 79% inhibition of 4 hour output of HCl as compared to control rats receiving saline. In both experimental series there was even greater inhibition in rats receiving juice from peptic ulcer patients. On the other hand, although carcinoma juice caused in both experimental series significant suppression of secretory activity, degree of inhibition was less than that produced by normal juice. Statistical analysis of the data showed that in both series of experiments the means of test groups were significantly different than those of control groups. Moreover, means of the "ulcer group" and "normal group" were significantly different ($p < .001$ in Series 1 and $p < .05$ in Series 2). Means of "normal group" and

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* This work was supported in part by Am. Cancer Soc. grant.

† Markle Scholar in Med. Sciences.

TABLE I. Effects of Gastric Juice from Normal Patients and from Patients with Gastric Carcinoma and Duodenal Ulcer on 4-Hour Gastric Secretion in Pylorus Ligated Rats.

	Controls, 1 ml saline	"Normal" juice, 6 mg/200 g	"Ulcer" juice, 6 mg/200 g	"Carcinoma" juice, 6 mg/200 g
SERIES 1	16 rats	16 rats	15 rats	16 rats
4 hr output of free HCl in meq	.509 ±.254*	.075 ±.063 p <.001	.030 ±.026 p <.001	.255 ±.223 p <.01
% deviation from controls		−85%	−94%	−50%
SERIES 2	15 rats	14 rats	14 rats	14 rats
4 hr output of free HCl in meq	.392 ±.322	.081 ±.089 p <.001	.029 ±.031 p <.001	.106 ±.083 p <.01
% deviation from controls		−79%	−92%	−73%

* Mean and S.D.

p = probability of significance of T value.

"cancer group" were significantly different only in the first series of experiments ($p<.01$ in Series 1 and $p<.20$ in Series 2). Means of "ulcer group" and "cancer group" were significantly different in both series of experiments ($p<.001$ in Series 1 and $p<.01$ in Series 2).

Discussion. Our data indicate that the active principle in human gastric juice responsible for inhibition of gastric secretion when given intravenously to rats is present in larger amounts or in a more active form in gastric juice from duodenal ulcer patients than in juice from normal individuals. On the other hand, this principle appears to be present in slightly lesser amounts or in a slightly less active form in gastric juice from individuals with gastric cancer. These results differ from those of Brunschwig and associates who found that gastric juice from patients with gastric cancer had greater inhibitory activity than juice from normal individuals(3). These investigators were injecting whole gastric juice in equal volumes, whereas in the present study equal weights of dialyzed, lyophilized juice were used. Gastric juice from patients with gastric cancer contains considerably more mucin per unit volume of water than does normal gastric juice. Therefore, it is likely that Brunschwig's results are explainable by much larger doses of the inhibitor substance in the dogs given carcinoma juice.

Assuming that this inhibitory principle has a physiologic role, it would appear that peptic ulcer disease is not due to a deficiency of this substance since it is found in peptic ulcer juice in greater amounts than in normal juice. However, since there is some evidence showing that the inhibitor is elaborated in the antrum(5), the greater inhibitory activity of peptic ulcer juice is in accordance with the fact that antral inhibition of gastric secretion is enhanced by hyperacidity of the antral contents. Conversely, one would expect to find less inhibitory substance in gastric juice under conditions of achlorhydria, as accompanying gastric cancer. However, the assumption that the inhibitory substance in human gastric juice, demonstrable by animal secretion studies, has a physiologic role in man has yet to be proven.

Summary. Intravenous administration of dialyzed, lyophilized gastric juice from normal subjects, patients with peptic ulcer and patients with gastric carcinoma, induced pronounced depression of secretion of hydrochloric acid and water in the pylorus ligated rat. Peptic ulcer juice had the greatest inhibitory effect, whereas gastric carcinoma juice had the least.

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Received June 8, 1960. P.S.E.B.M., 1960, v105.

LD-50 and Mitotic Rate of Animals at Time of Exposure to X-Radiation.*

(26067)

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It has long been known that rapidly growing tissues are the most susceptible to lethal effects of X-radiation. In studies of urethane carcinogenesis, a precise correlation was found between rate of growth of the pulmonary tissue in animals of varying age at time of injection of urethane and number of tumors initiated(1). It was further demonstrated that the variation in responsiveness between animals matched as to age, weight, sex, and strain and was directly related to the individual animal's growth rate at time of urethane injection(2). Growth rate of individual mice was estimated by number of mitoses occurring in unit lengths of ear epithelium biopsied immediately prior to injection, it having been previously demonstrated by Bullough in studies of diurnal variation (3) that though the absolute mitotic rate of various tissues was highly variable from tissue to tissue at a given time, mitotic rates in the various tissues rose and fell in concert. Therefore, it seemed worthwhile to find whether growth rate of individual mice at time of exposure to X-radiation would be related to whether they lived or died, that is, is such individual variation in growth rate the overriding factor determining the existence of an LD-50 under general laboratory conditions?

Methods and materials. "A" strain mice raised in this laboratory, but procured originally from Jackson Memorial Lab., Bar Harbor, Me., were matched as to age, weight, and sex. Immediately after biopsy of the ear of each mouse the animals were irradiated in groups of 8 in cages rotating at 11 rpm. The dose is indicated for each experiment in Table I. Multiple sections were made of each ear biopsy and number of mitoses occurring in a total of 4 cm of ear epithelium determined for each individual mouse. All recognizable states of mitoses were counted. Time of death for each of the animals was recorded for 30 days.

TABLE I. Mitotic Rate and Lethal Effect of X-Irradiation.*

	Avg mitotic rate†		No. of mice		
Group	Dead mice	Live mice	Dead	Alive	Total
A	10.54	4.6	19	11	30
B	11.5	5.1	27	13	30
25 ± 1 g mice, 697 r, approx. LD 65/30 da.					
C	9.7	5.1	11	29	40
17 ± 1 g mice, 600 r, LD 28/30 da.					

* Whole body irradiation 71 r/min.; 18 ma at 200 KV with ½ mm Cu and 1 mm Al filtration, T.S.D. 50 cm.

† Total No. mitoses in 4 cm ear epithelium.

Results. It is evident in Table I that the mice that died had considerably higher mitotic rates at time of irradiation than those that survived. No relation was found between mitotic rate of the individual mice and time interval to death. Variation in growth rate of groups of animals from cage to cage occurred which was uniformly associated with number of animals surviving in the individual cage.

These results make plain that relative mitotic rate of individual mice is an important factor in individual variation in susceptibility to lethal effects of X-radiation among mice matched as to age, weight, sex, and strain.

When put in terms of numbers of surviving or dying mice for various mitotic rates (Table II), it becomes evident that there are other factors more critical than growth rate. For example, 4 mice survived which had a growth rate higher than the mean of these that died and 10 mice died whose mitotic rate was lower than the mean of survivors.

These influences should be taken into account by those studying the mechanism of agents or environmental conditions relating to radiation protection. In addition, advan-

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* This work was done in the Depts. of Pathology and Radiology, Duke Univ. Medical Center, Durham, N. C., and supported by U. S. P. H. S. grant.

TABLE II. Distribution of Mice and Mitotic Rates of Mice that Died or Survived.

															Total
No. of mice dying	5	5	2	5	5	3	7	1	2	6	1	2	0	1	46
Mitotic count	0-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	
No. of mice surviving	10	5	4	1	0	2	1	1	0	0	0	0	0	0	24

tage might well be taken of the influence of diurnal variation in therapeutic utilization of X-radiation, for it is generally known that neoplasms do not undergo the degree of diurnal variation in mitotic activity that normal tissues do, remaining instead at a relatively high level.

Summary. A correlation is demonstrated between mitotic rate of individual mice at time of exposure and lethal effect of X-irradiation. The significance of this finding is discussed.

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Received June 15, 1960. P.S.E.B.M., 1960, v105.

Deposition of C^{14}-Labelled Cholesterol* in the Atheromatous Aorta.† (26068)

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This laboratory has been concerned with the relationship between cholesterol metabolism(1,2,3,4) and development of atheromatous conditions in patients. During investigation of the "biological halflife" of C^{14}-cholesterol in various patients, it was felt that the aorta might be permeable to circulating tagged cholesterol. For this reason 5 terminal patients received intravenous C^{14} tagged cholesterol and the aortas were assayed post-mortem to determine amount of C^{14}-cholesterol in various parts of the aorta, especially near the atheromatous plaques.

Methods. Radioactive labelled cholesterol-4-C^{14} (1-3 mg) obtained from a commercial source was dissolved in a minimal quantity of ethanol (0.3 ml). This cholesterol was introduced by use of a micro-syringe into a sterile ampule containing 2-3 ml of human serum albumin solution and a sterile glass-covered magnetic stirrer. While the stirrer was rotated by means of an external magnet, the alcoholic-cholesterol solution was slowly added. The sterile suspension of cholesterol in human serum albumin was used immediately after preparation(5). (The preparation has a tendency to settle after long standing.) This preparation containing the cholesterol (with 15 to 20 μc C^{14} radioactivity) was injected slowly (10 to 15 minutes) into the antecubital vein of selected patients. Terminal patients with life expectancy of from 2 to 10 days were selected for this study. These patients were aged and had clinical evidence of arteriosclerosis. Post-mortem examination revealed that all patients had well defined atheromatous plaques. Some of these plaques, in addition to being calcified, had adjacent ulcerations. Samples of the aorta of each individual were carefully obtained by use of a metal punch. Disks were obtained at intervals throughout the length of each aorta, representing an even distribution from upper to lower aorta (2 disks from the arch, 4 from thoracic region and 4 from abdominal region). Each disk was then dissected and the intima, media and adventitia separated. The separated intima and media sections of each disk were homogenized, treated with metaphosphoric acid and extracted continuously with ether in an automatic continuous extractor under nitrogen. The ether extracts were taken to dryness and the oil obtained dissolved in ethanol and treated with 1% solution of digitonin. The cholesterol digitonide obtained was repeatedly washed with the aid of centrifugation with acetone, ether and petroleum ether. The purified cholesterol digitonide was dissolved in methanol and assayed for radioactivity in a Packard "Tri-Carb" spectrometer with the aid of carbo-cell and a suitable phosphor (Popp). Blanks for radiometric assays were performed by homogenization of both intima and media disks of non-radioactive aortas similarly obtained. The results have also been corrected for background. Counting was done for a sufficiently long period to ensure a ± 0.3% counting error per determination. This probable per cent error has been determined by counting each sample to 30,000 total counts when the background was 25 counts/min(6).

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* All radioactive materials were obtained on allocation from U. S. Atomic Energy Comm.

† The authors are indebted to Drs. S. Bornstein, P. V. Skerrett, and H. T. Sugiura, Pathology Dept., for interest and cooperation in this study. We acknowledge technical assistance of E. S. Hercker, and statistical evaluation of data by J. S. Lafair.

TABLE I. Radioactivity Recovered* in Aorta of Patient "A" (Received 3-C^{14}-cholesterol 2 Days Prior to Expiration).

One inch diameter disks from aorta				Recovered radioactivity in cholesterol;† total cpm in disk	
Sequence No.		Aorta region	Atheromatous condition	Intima	Media
1	↑	arch	mild	875	380
2		"	severe	973	420
3		thoracic	"	812	430
4		"	medium	735	400
5	16″	"	plaque	239	530
6		"	medium	653	480
7		abdominal	"	685	435
8		"	"	227	385
9		"	"	295	430
10	↓	"	medium	842	480

* From 20 repeat determinations on aliquots of the same sample a spread of ± 12% was found for the experimental technics used.

† Cholesterol-digitonide precipitable material.

Results. In the media portion of the aorta of the patients studied, the recovered cholesterol showed a radiometric value approximately equal for each sample disk tested throughout length of aorta. The recovered cholesterol from the intima of the aorta gave a total radioactive value (counts/min.) for the region of the arch of the aorta that was slightly higher than for the disks obtained from other regions of the same aorta. In the areas where the punch included a large atheromatous plaque, values obtained showed a lower radioactivity value, thus indicating a lower exchange rate between cholesterol circulating and in the plaque. Values for patient A are presented in Table I. Results for radioactive assays of the disks of the other 4 patients were in all respects comparable to those obtained for patient A. In Table II are depicted average results obtained for the 5 patients studied. Our results are in some respects similar to those reported by Hollander(7). Cholesterol absorption in dog aorta has been reported to show a sharp reducing gradient going from thoracic to abdominal region. This gradient has also been observed in this study with human aortas. This gradient, however, was very gentle as seen by data in Table I. These results may be a consequence of a slower equilibration rate for the human aorta than the one observed in dog aortas(8).

The results conclusively show that circulating cholesterol can exchange with the available cholesterol pool of the aorta. Exchange or deposition on calcified structures (plaques) was slower than that found in non-calcified fatty deposits of the human aorta. The data were found to be significant for the assay used when submitted to standard statistical

TABLE II. Average Radioactivity Recovered in Aortas of 5 Patients (Received 3-C^{14}-cholesterol 2-10 Days Prior to Expiration).

One inch diameter disks		Recovered radioactivity in cholesterol; total cpm in disk	
No. of disks	Aorta region	Intima	Media
		± S.E.M.*	
10	arch	895 ± 25	430 ± 35
15	thoracic area	780 ± 37	400 ± 50
15	abdominal "	730 ± 50	380 ± 55
15	with plaques	240 ± 80	390 ± 66

* Stand. error of mean.

analysis (calculation of standard error of mean and Chi square analysis.)

Summary. The results suggest that circulating cholesterol exchanges with available cholesterol pools of intima of human aorta. This exchange is slow. Cholesterol in the atheromatous plaques appears to exchange or accept circulatory cholesterol with the greatest difficulty. The highest exchange or depositions were observed for area of the arch of human aorta; abdominal part of aorta showed a smaller exchange. The media showed about the same cholesterol rate of exchange through length of aorta. A disk technic suitable for various types of quantitative assays of intima, media, etc. of the aorta was described.

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Received June 20, 1960. P.S.E.B.M., 1960, v105.

Uptake and Excretion of Radioactive Rose Bengal Dye in Normal Dogs.*
(26069)

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Delprat demonstrated that rose bengal dye (tetra-iodo-tetra-chlorfluorescein) is rapidly concentrated by liver cells and subsequently excreted into the biliary tract(1). Since the initial studies on radioiodine^{131}-tagged rose bengal dye by Taplin and his associates(2), various investigators have utilized this substance for liver scintiscanning as a diagnostic procedure for detection of tumors, cysts and abscesses within the liver(3,4). Although initial studies on nonradioactive rose bengal dye suggested that little or no dye is concentrated by spleen, kidney or pancreas(1,5), the possibility remained that minute amounts might be taken up by these organs. Our studies were stimulated by a recent liver scan performed at this hospital in which a faint area of radioactivity was noted over the left upper quadrant of the patient's abdomen. Employing the radioactive form of the dye on rats, Glaser and co-workers recently concluded that "no large portion of the injected dye could be observed outside of the liver, blood stream, or intestinal tract(6)." To assess the role of various abdominal organs in concentration and excretion of even trace amounts of rose bengal dye, studies in normal dogs were undertaken. Our experimental findings indicate that in normal dogs uptake and excretion of the dye occur mainly through the hepatobiliary system and that pathways through other organ systems are negligible.

Method. Mongrel dogs, weighing from 5 to 10 kg, were anesthetized with 30 mg/kg of sodium pentothal. Using aseptic technic, the abdominal cavity was entered through a midline incision. During period of anesthesia, the dogs were maintained on a constant intravenous infusion of 5% dextrose in water. The animals were given 10% dextrose in water intravenously in experiments in which the urine was collected in order to assure an

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* Supported by grant from Minn. Division, Am. Cancer Soc.

† Postdoctoral research fellow under auspices of Nat. Cancer Inst.

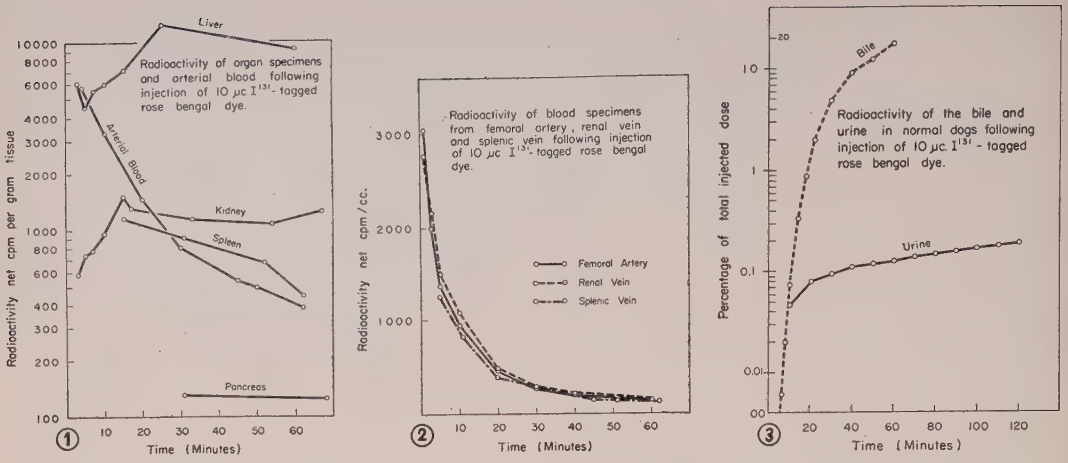

FIG. 1-3.

adequate urinary output during the study period. Ten μc of I^{131}-tagged rose bengal dye were then injected into a peripheral vein. In the initial study serial recordings on a rate meter were obtained for 60 minutes by holding a lead-shielded scintillation counter directly over various organs including liver, spleen, kidney, pancreas, duodenum, jejunum, and colon to determine gross changes in radioactivity during the acute experimental period. Using these recordings as a guide, subsequent experiments were performed, and serial biopsies of various organs obtained. Biopsies ranged in size from 125 to 825 mg in net weight and were obtained from liver, kidney, spleen, and pancreas in 5 animals. Splenic vein blood samples were obtained in 3 experiments *via* a small polyethylene catheter in one of the splenic veins near the hilus. Renal vein sampling was accomplished in 3 animals by threading a polyethylene catheter into the renal vein *via* the inferior vena cava thus minimizing any gross disturbance in renal hemodynamics. Serial arterial blood samples were collected in all animals with a catheter in the femoral artery. In 3 studies total urinary output was obtained by means of a bladder catheter. In addition, bile was collected in 2 animals through a polyethylene tube in the common duct. The duct was ligated distal to the catheter to divert totally the output of bile. Radioactivity of the various specimens, including the biopsy material, blood, urine and bile was determined immediately in a scintillation well counter for a minimum of 1,000 counts. Most specimens were counted for more than 10,000 counts.

Results. After injection of the dye, only small increases in radioactivity were observed over the kidney, spleen and jejunum when a scintillation counter was held over these organs. A slight increase in radioactivity was noted over the duodenum 40 minutes after radioactive rose bengal dye was administered. An equivocal increase in activity occurred at 55 minutes over the pancreas. It was felt that this represented duodenal radioactivity detected over the pancreas because of the proximity of the 2 organs and inadequate shielding of the counter to differentiate the two. A significant increase in radioactivity of liver was evident 4 minutes after injection of the dye and reached a peak in about 30 minutes.

Blood radioactivity after an initial elevation following injection rapidly decreased as the dye became concentrated by the liver (Fig. 1, 2). In 5 to 10 minutes, blood radioactivity fell precipitously to one half the concentration observed in the one minute sample.

Levels of radioactivity in splenic biopsies paralleled radioactivity of the blood stream, demonstrating the inability of the spleen to concentrate rose bengal dye (Fig. 1). Radioactivity of splenic vein blood appeared identical to that of femoral artery blood, also indicating lack of absorption of the dye by the spleen (Fig. 2). In the relatively avascular pancreas, radioactive levels in biopsied specimens were consistently lower than levels of the blood or spleen (Fig. 1). Interestingly, concentrations of dye in renal tissue appeared slightly higher than corresponding blood levels obtained at the same time (Fig. 1). However, a comparison of renal vein and femoral artery blood values of radioactivity failed to demonstrate appreciable withdrawal of dye from the blood by the kidney (Fig. 2).

Studies on excised hepatic tissue demonstrated concentration of the dye within 3 minutes after injection (Fig. 1). Peak levels occurred approximately 30 minutes after administration of the dye, when radioactivity concentration per gram of liver tissue was 10 to 15 times that of 1 ml of blood. By 60 minutes, the blood was rapidly cleared of dye and the liver/blood ratio was over 30.

Detectable levels of radioactivity were present in the bile as early as 6 minutes after injection of dye (Fig. 3). Concentration of rose bengal dye rose significantly during the subsequent 60 minutes. The reddish tinge of the dye was grossly apparent in the bile by about 10 minutes. At one hour approximately 20% of the dye had been excreted through the biliary tract.

A small, but measurable quantity of radioactivity was present in urine collected during the first 10 minutes after administration of dye (Fig. 3). Peak output of dye in the urine occurred 10 to 20 minutes after injection of rose bengal dye. Only very small quantities of dye could be detected in the urine subsequently. Total urinary output of dye at 60 minutes approximated 0.1% of administered dye. At 120 minutes, less than 0.2% of dye had been excreted in the urine.

Discussion. The present studies clearly indicate that the liver and biliary tract are the major sites of uptake and excretion of I^{131}-tagged rose bengal dye in the normal dog. In an animal with hepatic disease or biliary obstruction other pathways may assume greater significance. In the normal dog renal concentration and excretion appear to be insignificant. The greatest urinary output of the dye occurred during the same period of time that blood levels were highest. Urinary excretion may be due to blood levels elevated above renal thresholds. If this is so, the kidney may play a greater role in handling the dye in animals with hepatic or biliary disease.

In dogs, the spleen and pancreas do not appear to play an important role in handling rose bengal dye. In a patient without biliary obstruction, the presence of radioactivity in the left upper side of the abdomen after administration of the dye would most probably be due to an enlarged left lobe of liver. Since duodenal levels of radioactivity begin to rise within one hour, it is preferable to complete lower and midabdominal scanning before this period of time. For this reason scans in this hospital are started at the lower edge of liver, working upwards. Study of uptake and excretion was limited in these experiments to 1 to 2 hours since the acute period after administration of dye is of prime importance in performance and interpretation of hepatic scintiscanning.

Summary. In normal adult mongrel dogs, radioactive rose bengal dye is rapidly removed from the blood stream with radioactivity reduced 50% in 5 to 10 minutes following intravenous injection of the dye. The liver takes up the dye rapidly, starting within 4 minutes, and excretes it quickly, as evidenced by appearance of dye in the bile 6 minutes after administration. Approximately 20% of the tagged dye passed through the bile in one hour. Urinary excretion is almost negligible, with approximately 0.1% of total dye present in the urine in 60 minutes. Renal, splenic, and pancreatic tissue levels of radioactivity are insignificant and probably represent amount of dye in the blood stream. Radioactivity over the duodenum is evident within the first hour and probably represents amount of dye excreted into the bile.

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Received June 24, 1960. P.S.E.B.M., 1960, v105.

Leukocytic Transfer of Immediate-Type Hypersensitiveness in Man. V. Transfer of Experimental Sensitivity to Ascaris Antigen. (26070)

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Leukocytic transfer with peripheral blood leukocytes in an immediate type of hypersensitiveness was first demonstrated in man in the atopic form of sensitivity(1). The transfer reactions to pollens and danders in the recipients were specific but consistently small, never exceeding a slight, or one-plus (+), positive reaction. In more recent studies on experimental human sensitization with *Ascaris lumbricoides* antigen, marked transfer reactions of an immediate type were obtained in 2 noteworthy instances. These occurred in separate studies on donors who had been sensitized by repeated intracutaneous injections of *Ascaris* antigen and who exhibited, at time of transfer, delayed and immediate forms of hypersensitiveness to this antigen. In the first case, to be presented by Walzer, Bowman, and Coleman in another communication, both types of sensitivity were simultaneously and decisively transferred to a normal recipient with peripheral blood leukocytes or with extracts of these cells. In the second case described below, only the immediate type was carried over to a normal recipient by leukocytic transfer. The findings in this study represent the first instance of marked immediate-type transfer reactions elicited in man at sites prepared with peripheral blood leukocytes where only an immediate type of hypersensitiveness was transferred.

Materials and methods. The method of preparation and standardization of extracts of *Ascaris lumbricoides* worms removed from infested pigs has been described(5). Extract 11 prepared in 1954 and extract 12 prepared in 1959 were used in this study.

Donors, selected from members of the hospital medical staff, were young adults with a negative history of parasitic infestation. They were screened by an intracutaneous test with an *Ascaris* extract containing 0.01 mg N/ml and by tests with a few common allergens to rule out the presence of pronounced sensitivities. A positive family or personal history of atopy was not considered a basis for rejection, but individuals with active clinical symptoms of allergy were excluded.

The recipients in these experiments were young female student nurses and laboratory technicians with negative histories of parasitic infestation. They likewise failed to react to the screening test with *Ascaris* extract. Some were atopic, but none had active symptoms at time of this study.

To study the development of hypersensitiveness in the donor, intracutaneous tests were performed weekly with *Ascaris* extracts 11 and 12, using approximately 0.01 ml of the 0.01 mg N/ml dilutions for each test. Results were read in 15 minutes and graded according to accepted standards(6). At completion of these tests performed on the donor's right arm, a sensitizing injection of a mixture containing equal concentrations of extracts 11 and 12 was administered intracutaneously on his left arm. The delayed reaction produced by this injection was read in 24 hours. The horizontal and vertical diameters of the reaction were measured, and amount of induration was estimated by palpation and recorded in terms of 0 to ++++ (Table I).

In the successful experiment, the immediate-type sensitivity of Donor WT was transferred to Recipient BE. Donor WT is a physician, 26 years of age, who gave a posi-

TABLE I. Experimental Sensitization of Donor WT to *Ascaris* Extract and Results of Leukocytic Transfer Tests.

Day after screening test	Sensitizing injections				Delayed-type reactions (24 hr)		Immediate-type reactions			Results of transfer tests		
								Ascaris extract				
	Inj. No.	Amt, ml	Dilution, mg N/ml	Total dose, mg N	Size, mm	Induration	Test No.	No. 11	No. 12	Recipients	WBC sites	Serum sites
1	1	.05	.01	.0005	0	0	1	0	0			
10	2	.1	.05	.005	0	0	2	0	0			
17	3	.1	.1	.01	25 × 20	±	3	0±	±			
24	4	.1	.1	.01	20 × 20	+	4	±	±			
31	5	.1	.1	.01	15 × 20	+	5	0±	0±			
38	6	.1	.1	.01	30 × 30	++	6	+	±			
45	7	.1	.1	.01	50 × 50	++	7	+	+	DS	0	0
										BE	+++	0
										IL	0	0
52							8	+	+			

Scale of immediate-type skin reactions: + = wheal 0.5 cm in diameter with faint areola; ±, slightly smaller reaction; 0±, barely perceptible reaction; 0, negative reaction.

WBC = leukocyte sediment suspension (1:10).

Day 0, Feb. 7, 1960, day of screening test.

tive family and personal history of atopy and had no knowledge of any parasitic infestation. He failed to respond to screening tests with *Ascaris,* but showed weakly positive reactions to pollens and house dust. Recipient BE was a 17-year-old American-born female with a negative personal history of allergy, but her brother has hay fever. Her reactions to screening tests with *Ascaris* and with common allergens were negative, and she had no history of parasitic infestation.

The sensitization program carried out on Donor WT is presented in Table I. Delayed sensitivity was detected during the third week, soon followed by onset of immediate hypersensitiveness. Both forms developed slowly. By the seventh week, the immediate reaction to testing was no greater than a one-plus (+), and the delayed reaction following sensitizing injection was of moderate intensity.

A blood sample was drawn from Donor WT on day 45 before the skin tests for that day were performed. A sterile 1:10 suspension of leukocytic sediment in Tyrode's solution was prepared according to the previously described method(2) and was kept overnight in the refrigerator. On the following day, 0.05 ml of cell suspension was injected into each of 3 sites on the left arms of 3 recipients. On the right arm of each subject, 2 sites were prepared with Donor WT's serum derived from the same blood sample which provided the cells. After an interval of 11 days, the transfer sites were tested with 0.02 ml of *Ascaris* extract. Readings were taken in 20 minutes. Because leukocytic transfer reactions at this stage so closely resembled those regularly obtained at serum-sensitized passive transfer sites, the usual standards for reading the latter type of reaction(6) were also applied to the former.

Results. The 3 recipients were first tested on day 11 following transfer with a 0.01 mg N/ml dilution of *Ascaris* extract 12 and with a 0.05 mg N/ml dilution of extract 11. These tests were performed on 2 of the 3 leukocyte-injected sites on the left arm of each recipient, diluting fluid being used on the third site. In 2 recipients, results were completely negative (Table I). In Recipient BE, extract 12 injected into site 4 produced a moderate to marked positive (++±) reaction and extract 11 at site 5 elicited a marked positive (+++) reaction (Table II). The control test with diluting fluid on site 3 produced no reaction. When this site was retested 3 days later with an extract of ragweed pollen, an unrelated antigen, a negative reaction was obtained. A third test at site 3 on day 26 with *Ascaris* extract 12 elicited a positive (+) reaction. On the right arm, tests on day 11 at serum site 1 and at a control site with a 0.01 dilution of *Ascaris* extract 12 gave negative reactions.

On day 18, all points on both arms which

TABLE II. Results of Leukocytic Transfer Tests on Recipient BE.

			Right arm			Left arm			
Day after transfer	Test material	mg N/ml	Site 1 serum	Site 2 serum	Control	Site 3 WBC	Site 4 WBC	Site 5 WBC	Control
11	*Ascaris* 12	.01	0		0		++±		0
	" 11	.05						+++	0
	Diluting fluid			0		0			
14	Ragweed	.01				0			
26	*Ascaris* 12	.01				+			0

Blood drawn on donor day 45, as in Table I.
Transfer made on donor day 46, being considered recipient day 0.
WBC = leukocyte sediment suspension (1:10).
Scale of positive reactions: slight +; moderate ++; marked with pseudopods +++; excessively marked ++++.

had been injected with *Ascaris* extract developed faint nonpruritic, nonindurated erythematous spots from 2 to 5 mm in diameter. Sensitized and control sites on both arms reacted equally. The *Ascaris* tests performed on day 26 were followed on day 27 by similar reactions of no greater intensity. These reactions bore no resemblance to the severe delayed reactions seen in a previous experiment in which the delayed type of sensitivity was carried over with the immediate type. The faint reactions seen here probably represented a delayed type of hypersensitiveness originating in Recipient BE as a result of the previous tests with *Ascaris* done on day 11.

In this series of experiments, 2 other subjects were sensitized with *Ascaris* to serve as donors. One developed immediate and delayed sensitivities comparable in degree to those of WT, but a leukocytic transfer trial on a normal recipient proved unsuccessful. In the second subject, both types of sensitivity were only weakly positive and no transfer tests were attempted with his cells.

Discussion. In these studies, the immediate whealing type of specific response was confined to the leukocyte-injected cutaneous areas on the recipient and closely resembled in appearance the reagin-mediated reactions of serum-sensitized passive transfer sites(6). In comparison to the latter, however, leukocytic transfer reactions tended to develop more gradually, to be more intensively and persistently pruritic, and to manifest a more pronounced and prolonged edema.

The positive reactions elicited by testing the leukocytic transfer sites definitely exceeded those obtained at the serum-transfer sites as well as the donor's own responses to the same tests. This is a reversal of the relationhip observed in passive transfer phenomena produced with reagin-bearing sera.

In delayed-type hypersensitiveness, Lawrence found the degree of donor sensitivity to be a decisive factor in the success of leukocytic transfer tests(4). Pronounced sensitivity in the donor did not appear to be a prerequisite for leukocytic transfer of the experimental immediate-type hypersensitiveness to *Ascaris* seen in these studies.

The negative results obtained in Recipients DS and IL (Table I) who received experimental treatment similar to that afforded Recipient BE are indicative of the importance of still unidentified recipient factors in leukocytic transfer tests. In *Ascaris* sensitization studies, there is a particular need to keep in mind the possible influence of a previous, unrecognized parasitic infestation on subsequent immunologic responses of some recipients and donors.

Slight (+) leukocytic transfer reactions have been repeatedly elicited in the naturally acquired (atopic) form of immediate-type sensitivity(1,3). Marked (+++) leukocytic transfer reactions have thus far been obtained in 2 cases of immediate-type hypersensitiveness, the second of which has been described here. The cell donors in both instances had been experimentally sensitized to *Ascaris* and exhibited immediate and delayed reactions to this antigen at time of transfer. In both cases only one of several recipients tested showed positive transfer reactions. These findings, despite their limitations, render untenable the prevailing concept that only delayed forms of hypersensitiveness in man are transferrable by peripheral blood leukocytes.

Summary. From a human donor in whom immediate and delayed forms of sensitivity to *Ascaris lumbricoides* antigen had been experimentally induced, only the immediate type of sensitivity was transferred to one of 3 recipients by use of peripheral blood leukocytes. Markedly positive transfer reactions were obtained.

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Received June 30, 1960. P.S.E.B.M., 1960, v105.

Spontaneous Mammary Cancer Developing in Rats Born and Nursed by Mothers Treated with 3-Methylcholanthrene.* (26071)

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In the study of mammary carcinogenesis induced by 3-methylcholanthrene, one aspect of the investigation in this laboratory is concerned with the mechanism of chemical carcinogenesis. Development of mammary adenocarcinoma in rats following oral administration of 3-methylcholanthrene has been reported(1,2,3). These observations have indicated that the neoplasms induced by 3-methylcholanthrene were predominantly mammary cancers. The selective response of mammary tissue to orally administered 3-methylcholanthrene cannot be explained fully, since the mechanism of carcinogenesis is not known. However, Dao *et al.*(4) demonstrated selective concentration of administered polycyclic carcinogens in breast and fat and confirmed the earlier report by Shay *et al.*(5) of excretion of 3-methylcholanthrene into the milk of lactating female rats. These results raised certain questions: (a) If the chemical carcinogen can be excreted in the milk, could mammary cancer be induced in offspring suckled by mothers treated with 3-methylcholanthrene? (b) If 3-methylcholanthrene is administered to pregnant female rats, could it pass through the placental barrier, and induce, by its effect on the early embryo, mammary cancer in the offspring? (c) Can 3-methylcholanthrene induce a "product" which may be transmitted through the milk to the offspring, thus promoting tumor development?

The present article is concerned with some preliminary results of experiments undertaken in an attempt to answer these questions.

Materials and methods. In all experiments, 55- to 60-day-old mature female Sprague-Dawley albino rats were used. The animals were considered sexually mature when opening of the vagina and estrous cycles were established. Four groups of experiments were carried out: 1. 3-Methylcholanthrene was fed to the mature female rats at a dose level of 10 mg/ml sesame oil daily through a stomach tube for 6 days in one group and 10 days in the other. Each group consisted of 20 rats. Vaginal smears were taken daily after completion of feeding for 4 days to determine optimal time for mating. The female rats treated with 3-methylcholanthrene were then mated with normal males for a period of

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* This investigation was supported in part by grant of Nat. Cancer Inst.

TABLE I. Effect of 3-Methylcholanthrene on Litter Size and Survival of Newborn Rats.

No. of mothers	Duration of 3-MC adm. (days)	Litter size		No. newborn		No. surviving at weaning
		Range	Mean	Alive	Dead	
19	10*	3-12	6.5	85	12	6
14	6*	3-11	6.7	67	28	6
14	10†	5-12	8.8	122	3	7
16	6†	4-12	8.1	131	0	21

* 3-Methylcholanthrene, 10 mg/ml sesame oil, was fed to mothers during pregnancy.

† 3-Methylcholanthrene, 10 mg/ml sesame oil, was fed to female rats before mating with the males.

7 days. After the litters were weaned, female and male offspring were separated in different cages for long-term observation. These rats were examined at frequent intervals for palpable tumors. II. Sexually mature normal females and males were mated for one week. Vaginal smears were taken on the eighth day for a few days until pregnancy was ascertained. 3-methylcholanthrene was then fed to these pregnant rats at a dose level of 10 mg/ml sesame oil daily for 6 days in one group and 10 days in the other. After litters were weaned, male and female offspring were separated for observation of tumor formation. III. Normal male and female rats were mated. Five days after litters were born, 10 mg of 3-methylcholanthrene in 1 cc of sesame oil was fed to the lactating mothers daily for 15 days. Litters were weaned on the 21st day after birth. During the feeding, great care was taken to prevent ingestion of maternal feces by the infant rats. Mothers and offspring were housed in cages with bottoms of wide wire mesh that allowed the feces to drop from the cage. Also, these cages were frequently inspected to remove fecal material that failed to drop through the meshes. After weaning, female and male offspring were separated for observation. IV. Fresh mammary tumors removed from the rats were handled in a sterile manner and processed promptly. A 20% W/V tissue mince suspension in cold Ringer's solution was prepared. It was homogenized in an Omnimixer homogenizer for 10 minutes with the container immersed in an ice bath. The homogenate was centrifuged in a refrigerated centrifuge at 3000 G at 0°C for 30 minutes and supernatant fluid collected and passed through bacterial filters (Selas 02). *E. coli* (broth cultures) were added to the supernatant fluid prior to filtration to check the integrity of the filters.

Subcutaneously 0.1 ml of the cell-free filtrate was injected into the newborn rats less than 24 hours old. After weaning, male and female offspring were housed separately for observation.

Results. The effect of 3-methylcholanthrene on litter size and survival was summarized in Table I. When 3-methylcholanthrene was fed to the pregnant rats, incidence of stillbirth was high but it did not seem to affect the litter size significantly. When 3-methylcholanthrene was fed to the female rats some time before they became pregnant, incidence of stillbirth was greatly reduced. Survival of offspring of mothers treated with 3-methylcholanthrene was low in both situations. Most newborn rats died around 2 weeks after parturition; only 40 rats survived until time of weaning (21 days).

Results are summarized in Table II. Among a total of 28 surviving (7♂ and 21♀) offspring of mothers treated with 3-methylcholanthrene before pregnancy, 4 female rats developed spontaneous mammary cancers at the age of 49, 85, 90, and 90 days. These tumors were biopsied when they reached a size

TABLE II. Development of Spontaneous Mammary Carcinoma in Offspring of Mothers Treated with 3-Methylcholanthrene.

3-MC treatment of mothers	No. of mothers	Total No. surviving offspring ♂	♀	Appearance of tumors in daughters
Before mating	30	7	21	4/21
During pregnancy	23	3	9	1/9
After pregnancy, during lactation	10	9	11	0

of about 1 to 1.5 cm in diameter. Histologically, 3 are mammary adenocarcinoma and 1 an undifferentiated carcinoma. Only 1 mother had mammary cancer; the other 3 did not have tumors. One female rat among a total of 12 (3♂ and 9♀) offspring of mothers treated with 3-methylcholanthrene during pregnancy developed mammary cancer at the age of 200 days. Biopsy showed that it was cancer. In the experiment in which 3-methylcholanthrene was fed to the lactating rats, 20 offspring (11♀ and 9♂) from 10 lactating mothers survived for long-term observation. No tumors have been observed in these offspring at the end of 10 months. A total of 73 newborn rats was injected subcutaneously with a cell-free filtrate prepared from the mammary cancer induced with 3-methylcholanthrene. No mammary or other tumor has been detected at the end of one-year observation.

Discussion. We have not observed spontaneous mammary cancers in more than 1000 untreated female rats of the Sprague-Dawley strain used in this laboratory. Davis *et al.* (6) reported observation of spontaneous mammary cancer in 6 Sprague-Dawley female rats among a total of 250 animals observed over a long period. These tumors developed after the age of 540 days. Bullock and Curtis(7) reported observation of 521 spontaneous tumors in 489 animals from a total of more than 2450 rats autopsied. Among 521 spontaneous tumors, 94 were breast tumors, of which 7 were considered malignant and the rest were mostly benign fibroadenomas. Only 4 of these 7 malignant lesions were epithelial in origin, and they had appeared at 337, 354, 544, and 624 days of age. Benign fibroadenomas do occur in the breast of some of the female rats of several strains, but do not develop until the animals are very old(6,8). Furthermore, in those strains in which the females did develop malignant lesions in the breast, such tumors did not develop until well beyond the age at which they were manifested in these 4 Sprague-Dawley rats.

The mechanism by which these tumors develop is of considerable interest. Implication of genetics in the carcinogenic action of hydrocarbons has been made by many investigators(9,10,11). Strong(9) reported induced and spontaneous development of gastric lesions in mice. He observed that malignant gastric and other lesions were produced in descendants of mice treated with methylcholanthrene during several generations; these descendants themselves had never received any methylcholanthrene for 6 generations. Strong interpreted his results as due to a germinal mutation induced by the carcinogenic polycyclic hydrocarbon.

The meagerness of our present data does not seem to warrant any further interpretation. However, several possibilities can be considered.

First, these results may be interpreted as due to a germinal mutation induced by 3-methylcholanthrene, but this is a rather remote possibility. If this "mutational" change were germinal, one might expect a similar tumor incidence in both female and male offspring. Our results showed that the spontaneous tumors developed entirely in females and no tumors were observed in male offspring. However, it must be noted that since ovarian hormones play an important role in the induction of mammary cancers in rats treated with 3-methylcholanthrene, failure of development of spontaneous tumors in male offspring may conceivably be due to a lack of ovarian hormones.

The second possibility is a somatic mutation in the young receiving traces of 3-methylcholanthrene through the milk even days after feeding it to the mother. Our earlier results indicated that 3-methylcholanthrene could persist in the mammary tissues in measurable quantities for a considerable length of time(4,12). It would appear, therefore, that the mutation may have occurred in the embryonic stage of development. Spontaneous development of mammary cancer in a female offspring of a mother receiving 3-methylcholanthrene during her pregnancy indicates that the carcinogenic chemical is able to pass the placental barrier. This observation would favor the hypothesis that somatic mutation takes place in the embryonic stage of development.

Thirdly, it could be speculated that 3-methylcholanthrene induced a "product" which was transmissible through the milk to the offspring to cause spontaneous tumor development. The failure to induce mammary tumors in the rats receiving a cell-free filtrate prepared from the tumors induced by 3-methylcholanthrene does not seem to favor this speculation. Further investigations are now in progress.

Summary. Spontaneous mammary cancers were observed in 4 female rats among a total of 28 (7♂ and 21♀) offspring of mothers treated with 3-methylcholanthrene before pregnancy. These tumors developed at the age of 49, 85, 90, and 90 days. Histologically, 3 were mammary adenocarcinoma and 1 an undifferentiated breast cancer. One female rat among a total of 12 (3♂ and 9♀) offspring of mothers treated with 3-methylcholanthrene during pregnancy developed spontaneous mammary cancer at the age of 200 days. No mammary cancer was observed in rats whose mothers were fed 3-methylcholanthrene during lactation. Also, injection of cell-free filtrate prepared from the mammary cancer induced with 3-methylcholanthrene to the normal newborn rats of less than 24 hours old failed to induce mammary tumors in these animals. These observations are of great interest for further investigation.

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Received July 6, 1960. P.S.E.B.M., 1960, v105.

Estrogen Antagonisms: Effects of a Series of 19-Nortestosterone Derivatives on Vaginal Changes Induced by Estrone. (26072)

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Many chemical relatives of 19-nortestosterone are active as antagonists of uterine growth produced in mice by estrone and as progestational agents in Clauberg tests. There appears to be general correspondence between potencies in these tests, although no perfect correlation exists. Comparison of biological activity with chemical structure permitted conclusions regarding effects of structural alterations on activity(1). Since other studies had suggested remarkable differences in responsiveness of uterus and vagina to mixtures of steroid hormones(2,3,4,5), confirmation of these structure-activity relationships in vaginal smear tests became desirable. Comparable studies were possible only with discovery that progesterone would block estrogens administered over a 4-day period(4,6). This paper studies effects of derivatives of 19-nortestosterone as antagonists of estrone effects on the vagina.

Materials and methods. Assay methods employed here have already been described (4,6,7). Briefly, adult female Rockland mice were spayed and allowed 2 weeks to recover from effects of operation. They were then integrated into a colony and animals were chosen at random from colony for test-

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TABLE I. Comparison of Estrogen Antagonistic Effects of a Series of 19-Nortestosterone Derivatives and Other Miscellaneous Steroids in Vaginal Smear and Uterine Growth Tests. Under N, parenthetical number indicates number of tests in which a given compound was tested; number before parentheses is total number of smears upon which estimates were based. Code numbers refer to points on Fig. 1.

Compound or 17-substituent	Vaginal smear test N	Vaginal smear test Potency	Uterine growth, potency	Code No.
Progesterone	141 (4)	100	100*	1
Desoxycorticosterone acetate	101 (3)	42	10†	2
Testosterone propionate	165 (5)	200	70‡	3
19-nortestosterone	69 (2)	170	40*	4
Methyl 19-nortestosterone	61 (2)	1800	880*	5
Ethyl ”	92 (3)	2100	1250*	6
Vinyl ”	56 (2)	910	460*	7
Ethynyl ”	52 (2)	910	800*	8
Isopropyl ”	61 (2)	3100	2200*	9
n-Propyl ”	71 (2)	250	100–400*	10
Allyl ”	59 (2)	50	160*	11
n-Butyl ”	53 (2)	20	Inactive*	12
1-Methallyl ”	63 (2)	800	400*	13
2-Methallyl ”	61 (2)	530	680*	14

* Data from Edgren, Calhoun, Elton and Colton(1). † Data from Edgren and Calhoun (9). ‡ Data from Edgren and Calhoun(10).

ing each week. Test materials were administered in 0.1 ml per day of corn oil solution. Combinations of steroids were mixed and given in a single 0.1 ml injection. Each animal received 4 subcutaneous injections on mornings of first 4 days of test. Vaginal contents were sampled on afternoon of day 5 and morning of day 6. Unstained smears were scored according to method of Biggers and Claringbold(8), *i.e.,* an animal was scored as positive if there were no leukocytes in smear. Mice scored positive on day 5 were not studied again on day 6; a single positive smear sufficed to give any mouse a positive evaluation. Estrone was employed at a constant dose of 2 μg, and a series of doses of other steroid were injected simultaneously. Potency evaluations were based upon dose of blocker estimated to produce 50% depression in response to estrone. In most cases the 2 μg estrone dose stimulated positive responses in about 90% of mice. ED_{50} doses for blockers were read from graphs of estimated log dose-response curves.

Results and discussion. All compounds examined in this study were active (Table I), and there was a remarkable correspondence between results in this test and those obtained in uterine growth test. Again potency increased in the straight chain, saturated compounds from a low value of 170% for 19-nortestosterone itself to a high of 2100% for the ethyl; 3 and 4 carbon chains result in subsequent decreases for n-propyl and n-butyl. The latter shows some slight activity in this test, although it was not active as estrone antagonist in uterine growth test. Branching increased potency in this test; isopropyl, considered here as a branched ethyl, was more potent than ethyl, and both methallyls were more potent than allyl or n-propyl. The high degree of correspondence between results of this test and uterine growth test is shown in Fig. 1. Most compounds fell close to a straight line when potency in one test was plotted against potency in other test, indicating that both tests largely measured the same phenomenon. In terms of potency relative to progesterone standard, the compounds, with only 2 exceptions, were more potent as antagonists in vaginal smear than in uterine growth test. The 2 exceptions to this were allyl and 2-methallyl. Since the 1-methallyl acts much as one would predict from the remainder of series, there would seem to be no obvious reason why 2-methallyl gives an abnormal response pattern. It seems reasonable to assume that deviation of 2-methallyl is a chance occurrence and that only allyl is markedly divergent; why this should be is inexplicable at this time. Desoxycorticosterone acetate and testosterone propionate also show a close correlation between activities in this and uterine growth test.

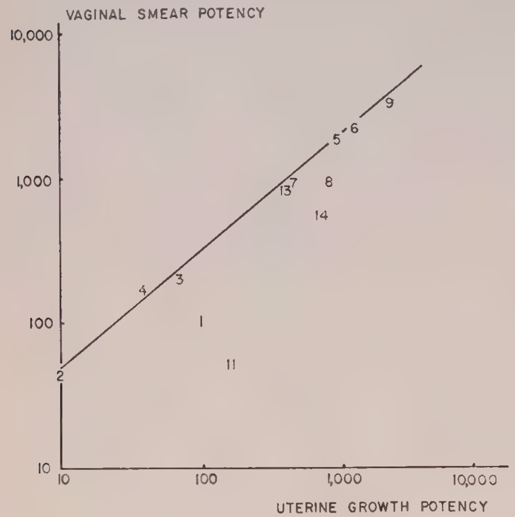

FIG. 1. Relationship of estrogen antagonistic potency in vaginal smear and uterine growth tests. Numbers refer to code numbers of Table I.

Summary. 19-Nortestosterone derivatives are antagonists of estrone in vaginal smear as well as uterine growth tests. Structure-activity relationships among these materials are largely the same in both tests.

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Received July 14, 1960. P.S.E.B.M., 1960, v105.

Suppression of LSD-25 Effects in Rats by Steroids.* (26073)

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Unpublished evidence by Resnick in our laboratories indicates that the effects of lysergic acid diethylamide (LSD-25) on smooth muscle contraction in an *in vitro* system may be altered by addition of steroid substances to the bathing medium of the muscle. This apparent antagonism is interesting to us because LSD-25 and steroids are known to affect the function of the central nervous system. Thus LSD-25 in small amounts produces a transient psychotic episode in man(1) and behavioral changes in animals(2). Likewise the balance of steroid hormones may alter mental function, *e.g.*, abnormal behavior may be seen both with adrenal insufficiency (Addison's disease) as well as excess (Cushing's disease)(3). Severe adrenal insufficiency in man is accompanied by a slowing of the electroencephalogram which is restored by cortisone(4). We have noted that adrenalectomy produces a significant decrease of approximately 10% in the dominant EEG frequency in rats and that this condition is corrected not only by most adrenal steroids but by the steroid hormone precursor Δ^5-pregnenolone as well(5). Δ^5-Pregnenolone has been shown to increase the efficiency level of a group of highly skilled motivated workers significantly above a similar group receiving placebos(6).

It occurred to us that investigation of the effects of steroids against behavioral changes produced by LSD-25 might show that steroids are effective anti-LSD agents *in vivo* as well as *in vitro*.

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* Aided by research grants from the Ford Foundation, G. D. Searle & Co., and N.I.M.H., U.S.P.H.S.

Materials and methods. The Winter and Flataker rat climbing time procedure was used as the bioassay method for this study (7). Young male Holtzman rats receiving limited food rations are taught to climb a vertical rope 5 ft. high for a food reward. Rats, when placed on a platform near the base of the apparatus learn to run quickly up the rope to a second platform containing dishes of food. After feeding for approximately 10 sec., the rats are returned to the cage. Well trained rats climb the rope in 2-4 sec., and this process can be repeated many times throughout the day with no evidence of decrement in performance as measured by the climbing time(8).

Compounds studied include the adrenal corticoids: desoxycorticosterone (DOC), dehydrocorticosterone, corticosterone, 11-desoxycortisol, cortisone and cortisol, the androgen and androgen metabolites: testosterone, Δ^4-androstenedione, androsterone, dehydroisoandrosterone and etiocholanolone, an estrogen: estradiol, and the C_{21} compounds pregnenolone, progesterone and pregnandiol.

One mg quantities of steroids contained in a vehicle of 25% propylene glycol and 75% physiological saline were injected intraperitoneally for 3 days prior to administration of LSD-25. On the test day the rats were injected with the steroid 1.5 hr prior to intraperitoneal administration of 40 γ LSD. Control rat groups received a similar series of injections of vehicle alone prior to LSD-25 administration. Climbing times for each rat were then obtained at intervals of 5, 10, 20, 30, 45 and 60 min. post injection in an alternating sequence. The performance of each animal injected with a steroid was compared with the performance of a vehicle injected rat. No difference has been noted between response to LSD of rats injected with vehicle and non-injected rats. Usually the group contained 5 rats each. Statistical evaluations were made using Wilcoxon's method for paired replicates(9).

Results. The effect produced by injection of 40 γ LSD-25 on climbing time is shown in Fig. 1. LSD-25 is rapidly absorbed and leads to a sharp rise in climbing time which reaches a maximum in 5-15 minutes followed by a slower return to normal climbing time. Evidences of LSD-25 action may still be apparent after 60 minutes but generally the effect has subsided (Fig. 2).

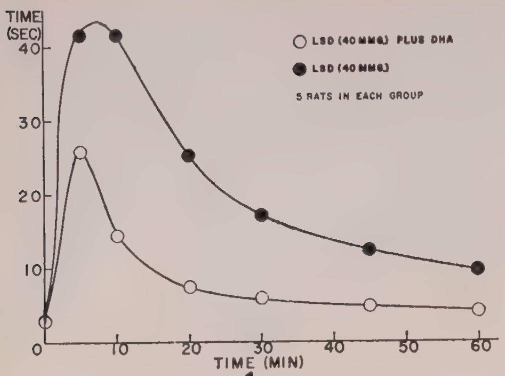
Injections of steroids alone during the 3 day pretreatment period produce no observable changes in climbing time. Mean values plotted in Fig. 1 and 2 for climbing times of a group of 5 rats treated with a steroid and a comparable group injected with the vehicle alone show no differences; pretreated and untreated groups have identical climbing times prior to LSD-25 administration.

Fig. 3 shows ratings of the various steroids tested for ability to suppress the action of LSD-25. Levels of statistical significance range from .05 to $<.001$.

Discussion. The ability of the steroids tested to suppress the LSD-25 effect in rats does not appear to be related to any specific molecular configuration. C_{19} compounds are as effective as C_{21} compounds under the test conditions employed. The negative results obtained with the single C_{18} steroid tested should not militate against the view that other C_{18} steroids may possess anti-LSD-25 activity. Presence or absence of hydroxyl or ketone groups in the molecule does not appear to be related to this activity. Likewise no correlation is evident between hormonal action or potency and anti-LSD-25 action.

The experiments with progesterone and LSD-25 in rats have been extended to a pilot human double blind study to be reported elsewhere. Briefly, 12 male volunteers were subjected to a battery of psychological tests found to be sensitive to LSD-25 in a previous study by Krus and Wapner(10). The test situation was designed to sample sensory-motor, perceptual and conceptual behavior. The results indicate that progesterone counteracts the effects of LSD-25 in some but not all of these tests, and that counteraction is not associated with a single behavior area: aberrations in all 3 behavior areas were corrected.

The mechanism of the action of steroids in rats and in humans has not been determined. The purpose of this study has been to screen



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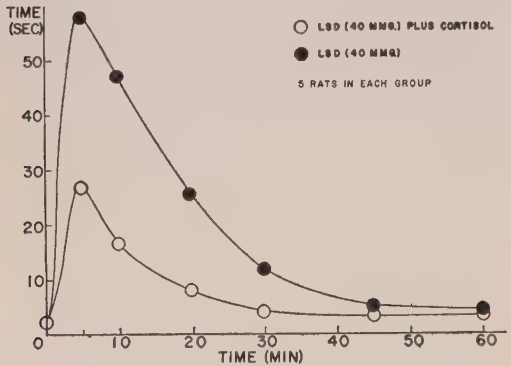

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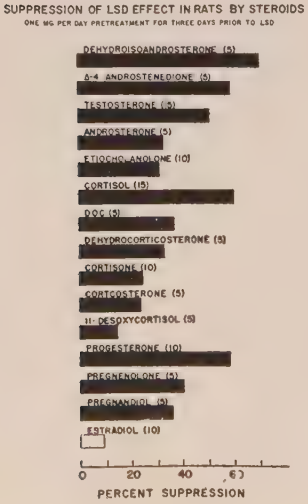

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FIG. 1 and 2. Effect on rope climbing time of rats inj. with LSD alone and in rats inj. with 1 mg dehydroisoandrosterone (DHA), Fig. 1, and cortisol, Fig. 2, for 3 days prior to LSD inj.

FIG. 3. Suppression of LSD effect on rat climbing time by steroids determined by comparison with an identical group of rats tested with LSD alone and tested simultaneously with each drug inj. group. Numbers of rats in each drug group shown in parentheses. An equal number of controls were used. Statistical significance denoted by solid bars, non-significance by open bar.

steroid substances for possible anti-LSD-25 effects. Future experiments will determine dose-response relationships for LSD-25-steroid interaction to obtain quantitative comparisons of the various steroids.

Summary. A series of 15 steroid hormones and metabolites were tested for their effectiveness to suppress LSD-25 induced behavior changes in rats. All produced significant suppression ($p<0.05$) except estradiol. Ability to suppress LSD-25 action does not appear to be related to specific molecular groups or to hormonal potency.

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Received July 15, 1960. P.S.E.B.M., 1960, v105.

Antiarrhythmic Activity of Various Steroidal Spirolactones in Dogs. (26074)

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The steroidal spirolactones have been of recent interest because of their ability to inhibit the urinary electrolyte effects of aldosterone and desoxycorticosterone (DOC) in laboratory studies(1-3) and in man(4-6). In addition, one compound in the series, SC-8109, appears to possess quinidine-like activity in isolated rabbit atria(7,8), and positive inotropic action on ventricular muscle(9). These observations prompted further studies on cardiac effects of steroidal spirolactones. The present report is concerned with antiarrhythmic properties of 3 aldosterone blocking agents, SC-8109, SC-5233 and Aldactone,* as determined by tests in anesthetized dogs. Three structurally related compounds with poor DOC-blocking properties were also chosen for cardiac tests, to assess the relationship of DOC-blocking and antiarrhythmic activities.

Materials and methods. Atrial arrhythmias were established in anesthetized adult mongrel dogs by crushing a portion of the atrium and stimulating electrically(10). This procedure usually resulted in atrial flutter with 2:1 A-V block. After a brief control period, compounds were given intravenously at 1 mg/kg/minute until a total dose of 20 mg/kg was administered, or until restoration of normal sinus rhythm(11). Spontaneous reversion to sinus rhythm occasionally occurred, but could be differentiated from drug action by reestablishment of flutter with immediate restimulation. If stimulation flutter could not be produced initially, local application of 0.05% solution of aconitine nitrate on the atrium produced atrial fibrillation(12). All compounds were given as 1.5 to 3.0% solutions in propylene glycol. The atrial electrogram, lead II electrocardiogram, and femoral artery blood pressure were recorded simultaneously. Typical reversions of each type of arrhythmia are shown in Fig. 1. The following compounds were tested: 3-(3-oxo-17β-hydroxy-19-nor-4-androsten-17α-yl)propanoic acid lactone, or SC-8109; 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)propanoic acid lactone, or SC-5233; 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)-propanoic acid lactone, or Aldactone; 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)acrylic acid lactone, or SC-4988; 3-(3-oxo-17β-hydroxy-19-nor-4-androsten-17α-yl)acrylic acid lactone, or SC-8288; and 3-oxo-17β-hydroxy-4-androsten-17α-yl propiolic acid, or SC-5314. Chemistry for most of these compounds has been reported(13-15).

Results and discussion. Table I summarizes results of our study in terms of mean effective dose (MED) for reversal of induced atrial arrhythmias in dogs. In our experience, the injury-stimulation arrhythmias are generally more difficult to correct than the aconitine type. That is, a compound with relatively weak antiarrhythmic potency may cause reversion of the aconitine fibrillation but may not appreciably affect a stimulation flutter. In view of the observed difference, results of the 2 tests are listed separately in Table I. Data on a standard antiarrhythmic drug, quinidine sulfate, are included in the table. It is noteworthy that aconitine arrhythmias responded to treatment with SC-8109, SC-5233 and Aldactone. SC-4988, a close structural derivative of SC-5233 with an unsaturated lactone group, also corrected aconitine arrhythmias in 4 dogs at a mean dose of 14.8 mg/kg. Comparison of MED in pairs by Wilcoxon Rank-sum test(16) revealed no significant differences among the 4 active compounds ($P>0.1$). In contrast, only SC-8109, the 19-nor compound, was effective against stimulation flutter; Aldactone, SC-5233 and SC-4988 were ineffective at 20 mg/kg. Three of the active spirolactones were also potent DOC-blocking agents, as shown in last column of table. The propiolic acid derivative (SC-5314) and unsaturated

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* Trademark of G. D. Searle & Co. for brand of spironolactone.

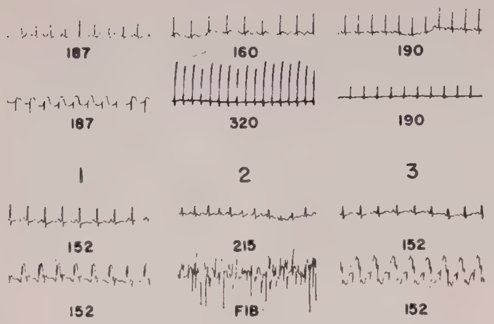

FIG. 1. (A) Stimulation flutter treated with DOC: 1. Control, before stimulation; 2. atrial flutter with 2:1 A-V block; 3. after 3 mg/kg DOC intrav. (B) Aconitine fibrillation treated with SC-8109: 1. Control, before aconitine application; 2. atrial fibrillation; 3. after 4 mg/kg SC-8109 intrav. Upper line in each record is lead II electrocardiogram and lower line is atrial electrogram. Number below tracing indicates ventricular or atrial rate in each case.

lactone in the 19-nor series (SC-8288), both with weak activity as anti-DOC agents, were without effect at 20 mg/kg. These last data could be interpreted as indicating some association between antiarrhythmic and DOC-blocking properties in steroidal spirolactones, but the relationship does not appear to be a strict one.

Additional comments may be made from the results of our study. The 19-nor modification is present in SC-8109 and SC-8288, but only the former was effective in correcting aconitine fibrillation. In addition, SC-5233 and Aldactone, which are members of the normal series, were approximately as effective as SC-8109. These observations indicate that antiarrhythmic activity is not directly dependent on 19-nor modification.

In the present study, DOC also reverses the atrial flutter resulting from electrical and aconitine stimulation (Table I), and shows, therefore, an effect similar to that of the spirolactones. This observation is in contrast to the antagonistic effects of these 2 types of compound with regard to kidney function. On the other hand, the data appear consistent with the results of *in vitro* experiments, in which spirolactones appear to act qualitatively like mineralocorticoids. Aldosterone, DOC, SC-5233 and SC-8109 were able to potentiate epinephrine-produced contractions of rabbit aortic smooth muscle(17). Quinidine, DOC and SC-8109 had similar effects on functional refractory period and trans-membrane potassium flux in isolated rabbit atria (7,8). Isolated papillary muscles from cat hearts gave positive inotropic responses when bathed with solutions of SC-8109 and DOC (9). These considerations suggest that the fundamental receptors affected by the 2 types of steroids may be identical in extra-renal tissues as well as in the kidney.

Summary. The effects of SC-8109, or 3-(3 - oxo - 17β - hydroxy - 19 - nor - 4 - androsten-17α-yl)propanoic acid lactone, and 5 structurally related compounds on atrial arrhythmias were investigated in anesthetized dogs. SC-8109 and 3 other steroidal spirolac-

TABLE I. Comparison of Anti-DOC and Antiarrhythmic Effects of SC-8109 and Some Structurally Related Compounds.

	Atrial arrhythmias				
	Stimulation flutter		Aconitine fibrillation		
Compound*	MED† (mg/kg)	Response‡	MED† (mg/kg)	Response‡	Anti-DOC potency§
SC-8109	14.5	2/2	8.3 ± 3.6‖	5/5	100
Aldactone	>20	0/2	10.2 ± 5.4	5/5	27
SC-5233	"	0/2	15.0 ± 4.6	5/5	41
SC-4988	"	0/3	14.8 ± 5.0	4/4	<4
SC-8288	"	0/1	>20	0/4	"
SC-5314	"	0/2	"	0/2	"
DOC	6	1/2	4.5	2/2	—
Quinidine sulfate	8.7 ± 5.8‖	8/8	8.6 ± 4.2	14/14	—

* Propylene glycol, the solvent used for all compounds, was inactive in 6 arrhythmic dogs at 12 ml/dog.

† MED = Mean effective dose.

‡ Response = Ratio, No. of reversions : No. of dogs tested.

§ Relative parenteral potency calculated from data supplied by Dr. C. M. Kagawa.

‖ ± stand. dev.

tones (SC-5233, Aldactone and SC-4988) were approximately equipotent in correcting aconitine-induced arrhythmias. Of these compounds, only SC-4988 did not possess significant DOC-blocking activity in the mammalian kidney. The other 2 compounds in the series, similarly weak as DOC-blockers, were inactive as antiarrhythmic agents. SC-8109 alone reversed the arrhythmia induced by electrical stimulation. DOC and quinidine sulfate were effective against both types of flutter. The relationships of structure-antiarrhythmic activity and of DOC blocking-antiarrhythmic activities of the steroidal spirolactones are discussed.

The author is indebted to Dr. C. M. Kagawa for many valuable suggestions, to Miss Marlene Shepard for technical assistance, and to Mr. D. W. Calhoun for statistical aid.

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Received July 15, 1960. P.S.E.B.M., 1960, v105.

Influence of Hair Growth Cycle on Lipid Composition of Mouse Epidermis and Dermis.* (26075)

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Recent evidence indicates that the hair growth cycle in the mouse not only influences the thickness and morphology of the entire skin, but also has some effect on its chemical composition. Changes in chemical composition of skin as related to hair follicle activity (1,2,3) are mainly obtained from histochemical procedures(4). Little is known concerning alterations in lipids in skin during the hair growth cycle. Although the adipose layer of skin doubles in thickness during periods of active hair growth, total fat content is not significantly increased over that in skin containing quiescent hair follicles(3). Lipid composition of epidermis and dermis including adipose and muscle layers for skin in growing (anagen VI) and quiescent (telogen) hair follicles(5) is given in this report.

Methods. Hair growth was initiated in adult Swiss mice by plucking of quiescent (telogen) hair from the entire dorsum of each mouse(5). Skin samples were obtained from mice killed by cervical dislocation on the 12th (anagen VI) and 22-23rd (telogen) days following plucking(5). Fat below the panniculus carnosus was carefully removed after which the epidermis was separated from the dermis at 50°C by the procedure of Baumberger *et al.*(6). Dermal specimens included the adipose layer and panniculus carnosus. The variation in dermal preparations produced by scraping of these layers from

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* Aided in part by grant from Nat. Cancer Inst., U.S.P.H.S.

the dermis was thus avoided. For analysis the epidermis from 50 to 100 mice and dermis from 6 to 10 animals were used. These tissues were stored under alcohol at a –25°C until ready for extraction.

Total lipid was extracted from the epidermis and dermis at room temperature by the procedure of Hanahan *et al.*(7). To facilitate extraction of dermis, it was homogenized in 120-160 cc alcohol at full speed (Omnimixer) then stirred for 4 hours at room temperature. The residue from the ethanol extraction was subsequently homogenized in 160 cc alcohol-ether 3:1 V/V as above and stirred for 3 hours. Final extraction was accomplished by stirring the residue again for 3 hours in 160 cc of the alcohol-ether solution. The fat-free residue of the tissue was dried overnight at 110-115°C and weighed. Solvents were removed from the lipid extract under vacuo over N_2 at 40-50°C(7). Phospholipids were then separated from the total lipid mixture with acetone(7). Acetone soluble material was dissolved in petroleum ether (60-70°C) and 300 mg were fractionated on silicic acid by the procedure of Hirsch and Ahrens(8). This method gave the cholesterol esters, triglycerides and cholesterol. Fast acting sterols were estimated on some of the epidermal cholesterol and cholesterol ester fractions by the procedure of Moore and Baumann(9), cholesterol by the method of Abell *et al.*(10) and triglycerides by weight. The iodine number of triglyceride fatty acids was determined by the micro absorption Wijs method after Luddy *et al.*(11).

Results and discussion. Hair follicles in a quiescent or actively growing state were without effect upon per cent water, total lipid, phospholipid or neutral lipid content of either dermis or epidermis (Table I). The water and neutral lipid content of epidermis is lower and phospholipid content higher than that of dermis. The slight effect of the hair growth cycle on skin water content is in contrast to the findings of Butcher and Crokoest who found a correlation between growth of hair follicles and water content in rat skin (12). Cholesterol and cholesterol ester content of anagen and telogen epidermis is about the same and considerably greater than that of anagen and telogen dermis which have comparable amounts of these constituents (Table II). The fast acting sterols of the epidermis are limited essentially to the cholesterol ester fraction of which they are a considerable proportion, in agreement with the observations of Kandutsch *et al.*(13) and Frantz *et al.*(14). There is no significant difference in other lipid components between anagen and telogen dermis or between anagen and telogen epidermis. The lack of salient chemical differences in epidermis or dermis during anagen and telogen stages of the hair growth cycle seems most unusual in view of the findings of Chase *et al.*(15), who showed that in skin of C57BL/6CH strain mice the corium and adipose layers during the anagen stage of the hair growth cycle are respectively 800 and 300 μ thick whereas in the telogen phase these layers are 400 and 200 μ thick, respectively.

Summary. Water and lipid composition of epidermis and dermis (including adipose and muscle layers) were determined during the anagen VI (growing) and telogen (quiescent) stages of the hair growth cycle. These stages

TABLE I. Water and Lipid Content of Dermis and Epidermis as Influenced by Hair Growth Cycle.

Tissue		Water	Total lipid	Phospholipid	Neutral lipid
		%			
Dermis-anagen*	(5)	64.8 ± 3.1	16.4 ± 1.6	7.8 ± 2.1	92.2 ± 2.0
Epidermis-anagen*	(4)	54.1 ± 2.7	19.9 ± .7	13.9 ± 1.5	85.1 ± 1.5
Dermis-telogen†	(5)	62.6 ± 3.4	19.0 ± 1.5	6.3 ± 1.5	93.7 ± 1.5
Epidermis-telogen†	(4)	53.7 ± 1.0	23.6 ± 1.2	11.8 ± 1.2	88.2 ± 1.2

* Anagen-dermis and epidermis removed from skin of 12th day of hair growth cycle.

† Telogen-dermis and epidermis removed from skin of 22-23rd day of hair growth cycle.

No. of samples analyzed indicated in parentheses. Epidermis of 50 to 100 mice was used for each analysis.

TABLE II. Lipid Composition of Epidermis and Dermis as Influenced by Hair Growth Cycle.

Tissue		Cholesterol esters, %	Cholesterol, %	Triglycerides	Iodine value, Wijs
Dermis-anagen*	(9)	.27 ± .06	.75 ± .2	99 ±.16	71 ±13
Epidermis-anagen*	(5)	2.3 ± .6 †	3.1 ± .5	95 ±.6	59 ± 3
Dermis-telogen*	(6)	.44 ± .09	.5 ± .14	99.1±.2	63 ± 8
Epidermis-telogen*	(4)	1.8 ± .4 ‡	2.1 ± .4	96 ±.4	62 ± 4

* Anagen and telogen dermis and epidermis as indicated in Table I. No. of samples analyzed indicated in parentheses.

† Fast acting sterols were 18% of esters, 9.4% of total steroids.

‡ " " " " 27% " " , 13% " " " .

of the hair growth cycle represent times of maximum morphological differences in thickness of the corium and adipose layers. Yet total lipid, phospholipid, neutral lipid, cholesterol esters, cholesterol and triglyceride content of epidermis during anagen VI and telogen stages of the hair growth cycle are not significantly different. The same is true for the lipid composition of the dermis. However, independent of hair growth activity, epidermis has a lower water content and a much greater level of cholesterol, cholesterol esters and phospholipid than dermis. The fast acting sterols of epidermis are limited largely to the esterified fraction.

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Received July 18, 1960. P.S.E.B.M., 1960, v105.

A Heat-Stable Hemagglutinin in Mouse Tissue Extracts.* (26076)

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It has been observed repeatedly that hemagglutination by the polyoma virus takes place at "refrigerator," but not "room," temperature(1). In our laboratory we have been investigating the possible presence of this agent in normal and neoplastic mouse tissues, using hemagglutination *in vitro* as a method of identification(2). This paper describes a heat-stable hemagglutinin, derived from extracts of mouse tissues and active at both 4° and 23°C, which was demonstrated in the course of these experiments.

Materials and methods. Four kidneys, 26 livers, 2 lungs and 6 mammary glands were obtained from 34 mice of 20 inbred strains.

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* Supported in part by a grant from The Leukemia Society, Inc., and in part by grants from Nat. Cancer Inst., N.I.H., P.H.S.

† Post-doctoral fellow, 1959-60, supported by a grant from Nat. Inst. Health, P.H.S.

TABLE I. Agglutination of Mouse Red Blood Cells by Tissue Extracts (Fraction 1, Fraction 2 and Fraction 3).*

		Titers†			
		With nonheated extracts		With heated extracts	
Tissue extracted	Extracts*	4°C	23°C	4°C	23°C
Normal mouse	Fraction 1	0	0	80–320	80–640+
	" 2	0–40	0–40	0–80	0–320
	" 3	10–40	10–320	10–160	40–640+
Mouse tumors	" 1	0–20	0	0–320	0–640+
	" 2	0	0	0–80	0–320
	" 3	20–160	20–160	40–640+	160–640+
Beef liver, kidney; lamb liver	" 1	0–640+	0–320	80–160	20–160
	" 2	0	0	0–160	0–160
	" 3	640+	20–80	80–160	40

* Fraction 1, supernatant after 550 × g; Fraction 2, supernatant after centrifugation of Fraction 1 at 105,000 × g; Fraction 3, pellet after centrifugation of Fraction 1 at 105,000 × g.

† Titers shown as reciprocals of extract dilutions. 0 = no agglutination. Red cell-extract mixtures incubated at temperatures shown.

Thirteen autochthonous mammary adenocarcinomas, 5 autochthonous leukemias, 2 transplanted leukemias, a transplanted melanoma and a transplanted squamous cell carcinoma were obtained from a hybrid mouse and 21 mice of 7 inbred strains. Tissue extracts were prepared as follows: a 10% suspension of tissue in Hank's solution was homogenized with a glass homogenizer and motor-driven Teflon pestle. The homogenate was spun at 550 × g for 10 minutes in an International refrigerated centrifuge (Model PR-1). An aliquot of the supernatant was removed (Fraction 1) and the remainder spun at 105,000 × g for 35 minutes in a Spinco Model L centrifuge. The supernatant (Fraction 2) was saved and the pellet (Fraction 3) resuspended (1.0 ml/g of original tissue) in phosphate buffered saline (pH 7.2). Materials were handled aseptically and kept chilled at all times. All tissue extracts were stored for varying intervals at –60° to –70°C prior to testing. Whole blood, obtained from young C57BL/Ks mice by tail bleeding and from adult guinea pigs by heart puncture, was collected in Alsever's solution. Red blood cell suspensions were prepared by the method described by Hartley *et al.*(3). Hemagglutination tests were run in Kahn tubes using serial 2-fold dilutions of tissue extracts in buffered saline (pH 7.2). Each tube, containing 0.2 ml of the diluted extract, received 0.2 ml of a 1.0% suspension of washed red cells. Both nonheated and heated preparations (56°C for 30 minutes) were used. The tests were carried out at room temperature (23°C) and in the cold (4°C); readings were made after 2 to 12 hours by the pattern method.

Results. Agglutination of mouse erythrocytes by normal mouse organs. Table I summarizes data obtained when extracts of normal mouse organs were tested for agglutinating action with mouse red cells. The agglutinating effect could be altered by several factors. Nonheated preparations yielded similar titers when incubated at 4° or 23°C, but were less active than heated preparations (56°C for 30 minutes) at either incubation temperature. The highest titers were obtained with heated extracts incubated at 23° C. The importance of centrifugation is shown by a comparison of agglutination titers obtained with the 3 tissue extracts. In general, Fraction 1 and Fraction 3 gave higher titers than Fraction 2. These results suggest that the heat-stable agglutinin does not sediment with low-speed centrifugation (500 × g for 10 minutes), but is partially sedimented by centrifugation for 35 minutes at 105,000 g. Some variability in titer was noted when comparisons were made among extracts of the different organs. Highest titers were obtained with liver and lowest with mammary gland. Comparable titers were observed with extracts of lung and kidney; these values occupied a median position.

Agglutination of mouse erythrocytes by *mouse tumors.* Titers of extracts of mouse neoplasms incubated with mouse red cells were comparable to those observed with normal mouse organs (Table I). Further, similar effects of centrifugation, incubation temperature, and heating on titer were noted. Titers with extracts of transplanted tumors (leukemias, squamous cell carcinoma and melanoma) were low. Those observed with autochthonous leukemias and mammary adenocarcinomas were similar to the titers found with normal liver extracts.

Agglutination of mouse erythrocytes by extracts of beef and lamb tissues. Consideration was given to the possibility that the heat-stable agglutinin might be present in tissues of species other than the mouse. Beef liver and kidney, and lamb liver were supplied by a local market; tissue extracts were prepared as above. Titers were obtained with these preparations, but they were quite variable (Table I). Centrifugation and temperature of incubation did not alter the agglutinating effect in a predictable fashion. In contrast to results obtained with mouse tissues, heating at 56°C for 30 minutes effected a reduction, rather than an increase, in titers. Considerable variability in titers was also evident when comparisons were made among the different tissue fractions.

Agglutination of guinea pig erythrocytes. Tests for agglutination of guinea pig red cells by tissue extracts (normal mouse organs, mouse tumors, beef and lamb tissues) were carried out on a representative number of extracts. Routinely, the titers were low when compared with those obtained with mouse erythrocytes. Extracts of mouse tumors had little or no agglutinating effect, but in general, the results reported above with mouse erythrocytes are applicable here.

Effect of filtration on agglutination. Low-speed supernatants (Fraction 1) of mouse tissues, made as described previously, were prepared from normal C57BL/Ks livers, autochthonous mammary adenocarcinomas from A/Sn and ST/Ks donors, and AKR/J donors with autochthonous leukemias (liver, spleen, thymus, lymph nodes). Aliquots were saved and remaining supernatant materials filtered (Selas porcelain candle, No. 03). The agglutinating action of these extracts on mouse erythrocytes is summarized in Table II. The effect of heating on agglutination titers is evident. Heated filtered extracts had a lower agglutinating effect than heated nonfiltered preparations (Fraction 1) at either incubation temperature.

TABLE II. Agglutination of Mouse Red Blood Cells by Tissue Extracts (Fraction 1 and Filtrate).*

Tissue extracted	Extracts*	Titers† With nonheated extracts 4°C	23°C	With heated extracts 4°C	23°C
Normal mouse liver	Frac. 1	0	0	320	320
	Filtrate	0	0	80	160
Autochthonous mammary ca.	Frac. 1	0	0	320	320
	Filtrate	0	0	20	20
Autochthonous leukemia	Frac. 1	80	40	320	160
	Filtrate	0	0	80	40

* Fraction 1, supernatant after 550 × g; Filtrate, prepared by filtration of Fraction 1 (03 Selas porcelain candle).

† Titers shown as reciprocals of extract dilutions. 0 = no agglutination. Red cell-extract mixtures incubated at temperatures shown.

Failure to reverse agglutination. Mouse tissue extracts and mouse erythrocytes were incubated at 4°C for 2 hours. Titers were recorded, and the cells were then washed 3 times in chilled buffered saline (pH 7.2). Supernatants of the third washing were drawn off and mixed with fresh red cells; the remaining sedimented cells were resuspended in fresh saline. Both sets of tubes were incubated at 37°C for 30 minutes, then at 4°C for 2 hours. There was no reversal of agglutination; approximately 15% of the heat-stable agglutinin was eluted.

Variable ranges in agglutination titers. Consistent titers with a given sample were observed in all but a few instances. Negative results were obtained from experiments designed to test the possibility that repeated freezing and thawing of the tissue extracts or aging of the red blood cells might account for the occasional variability.

Discussion. Salaman(4) described hemagglutination of mouse erythrocytes by extracts of normal and cancerous mouse tissues. Later, Gross(5) reported that mouse red blood cells are agglutinated by passage A leukemic filtrates. The present study extends these experiments by demonstrating the presence of a heat-stable agglutinin in extracts of normal, as well as neoplastic mouse tissues. The identity of the heat-stable agglutinin has not been ascertained, nor has it been possible to determine whether the activity of these preparations is due to more than one agglutinating agent. However, the data support the concept of a hemagglutinin which is not a tumor virus. The heat-stable agglutinin differs from the polyoma virus (*cf.*(6)) in 3 ways: (a) only a slight elution (approximately 15%) of the heat-stable agglutinin occurs at 37°C; (b) mouse red cells give higher agglutination titers than guinea pig erythrocytes; and (c) agglutination takes place both in the cold (4°C) and at room temperature (23°C).

Fundamental differences exist between the heat-stable agglutinin and the hemagglutinating agents reported by Salaman(4). Significant differences are not apparent, however, when a comparison is made between the agglutinating action of passage A leukemic filtrates(5) and our mouse tissue extracts, whether of normal or cancerous tissues. Gross(5) poses the question as to "whether the agglutinating potency of leukemic extracts is actually related to presence of the leukemic agent." Comparison of our results obtained with filtered and nonfiltered extracts of mouse tissues shows that filtration effects a marked reduction in agglutination titers. These findings are contrary to what one would expect if a viral agent were involved.

The agglutinating activity which we observed with extracts of beef and lamb tissues was not anticipated. The data indicate that agglutinin(s) in the beef and lamb preparations is neither a serum antibody, since high titers were obtained with Fraction 3, nor analagous to the heat-stable agglutinin derived from mouse tissues. In addition, these observations point out the need for caution before stating that the agglutinating action of a given tissue extract is due to a tumor virus.

Summary. Extracts of normal and neoplastic mouse tissues were found to agglutinate mouse and guinea pig red blood cells. Characteristics of the heat-stable substance(s) responsible for this phenomenon are discussed and it is proposed that the hemagglutinating agent(s) is not a tumor virus.

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Received July 18, 1960. P.S.E.B.M., 1960, v105.

Urobilinogen Excretion After Hemoglobin Infusions in Patients with Normal Hematologic and Hepatic Findings.* (26077)

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According to Watson(1), the normal daily excretion of urobilinogen in feces ranges from 40 to 280 mg, averaging 140 mg in a recent study. In the urine, daily normal excretion is from 0.5 to 1.5 mg. Theoretically, it is possible to calculate rate of hemoglobin destruction from daily excretion of urobilinogen on the assumption that 1 mg of urobilinogen is derived from 28.5 mg of destroyed he-

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* This study was supported in part by grants from Nat. Cancer Inst., U.S.P.H.S. and Am. Cancer Soc.

moglobin. Thus, a daily total excretion of 140 mg of urobilinogen would represent the catabolism of 3.99 g of hemoglobin. However, this corresponds to less than ⅔ of the approximately 6.25 g of hemoglobin assumed to be destroyed daily. In view of this discrepancy, it was considered of interest to measure the increment in fecal and urinary urobilinogen following slow intravenous infusion of a known amount of hemoglobin over a period of a few hours.

Methods. Subjects. Six patients in their sixth to ninth decades but with no known hematologic disorder and hemoglobin levels of above 14 g% were selected for study. Cases 2, 4 and 5 were confined to bed, the others were ambulatory about the hospital ward. Their liver function tests such as bromsulfalein, cephalin flocculation, thymol turbidity and alkaline phosphatase were normal, as was their subsequently observed urinary excretion of urobilinogen during control periods.

Hemoglobin solution. Fifty to 100 ml of venous blood were drawn from the patient or from a normal individual with compatible blood and mixed with 500 ml of sterile distilled water. The hemolyzed blood was centrifuged and the supernatant introduced into 1000 ml of sterile 1.25 g% NaCl, making a final tonicity of approximately 0.83 g%. Amount of hemoglobin/100 ml of prepared solution was determined by using an Evelyn photoelectric colorimeter.

Hemoglobin infusion. The freshly prepared hemoglobin solution was given to the patient intravenously through an adjustable, constant rate infusion pump(2) at from 10 to 16 mg of hemoglobin/min in amounts ranging from 7.80 to 11.75 g in different patients. In 1 patient, who received the largest amount of hemoglobin, an infusion was given on each of 3 consecutive days; in 4 patients duration of infusion was from 4 to 5 hr on 2 consecutive days; in 1 patient, 9 hr in 1 day. Blood samples diluted 9:1 with 3% sodium citrate solution were collected just before and at the end of each infusion as well as 3 or more times before and at least 5 times after infusion at intervals of 1 to 2 days for 8 days or more. Determinations of plasma hemoglobin and bilirubin levels were made on these samples using the methods described by Ham(3) and by Ducci and Watson(4), respectively.

Stool collections. These were made over 4-day periods, 5 or more times before, and 4 or more times after beginning of infusions. Before the first 4-day collection of the control period and also on resumption of successive 4-day collection periods after any interruption of the series, 500 ml of saline or tap water were given to the patient as an enema. This was also done just before hemoglobin infusion. Fecal material so recovered was discarded. Otherwise, all stools were collected in 8 x 7 inch glass jars with covers and placed in an insulated metal container half-filled with ice and covered tightly. Determinations of urobilinogen were made by the method of Schwartz *et al.*(5) as soon as each 4-day collection was completed.

Urine samples. These were collected as 24-hr samples for at least 5 consecutive days before infusion. During day of infusion, all urine secreted was collected and analyzed as separate samples. Thereafter, 24-hr urine samples were collected again for at least 5 consecutive days. The accumulating samples of urine were stored in a refrigerator until the 24-hr collection was completed, when determinations of urobilinogen and hemoglobin were made immediately. Standard urinalyses were also performed.

Results. Table I presents the data on fecal urobilinogen excretion during 4-day collection periods before and after beginning of hemoglobin infusions in the 6 patients. Table II shows amounts of hemoglobin theoretically catabolized to produce the observed amounts of urobilinogen excreted both during 8 days of control periods and during the 8 days following start of hemoglobin infusions. The percentage efficiency of the conversions of catabolized hemoglobin to urobilinogen is also shown.

Since the fecal urobilinogen recovered after infusion increased most in the first 4-day period, to a lesser degree in the next 4-day period, and not perceptibly thereafter, average daily fecal urobilinogen before infusion of hemoglobin x 8 was subtracted from total amount of fecal urobilinogen excreted during the first 8 days after infusion. The difference was taken as total increase in fecal urobilinogen excretion resulting from the hemoglobin infused. The total resulting increase in urinary urobilinogen excretion, which usually lasted from 4 to 8 days but was very small (2.4 to 11.4 mg), was not taken into account in the calculations.

None of the injected hemoglobin appeared

TABLE I. Fecal Urobilinogen Excretion (mg) during 4-Day Periods before and after Hemoglobin Infusions in 6 Patients.

Collection period	Case 1 ♂, 66 yr, cancer of prostate	Case 2 ♂, 79 yr, cancer of prostate	Case 3 ♀, 70 yr, cancer of breast	Case 4 ♂, 82 yr, multiple sclerosis	Case 5 ♂, 61 yr, chronic arthritis	Case 6 ♂, 55 yr, muscular atrophy
1	204	111	163	180	267	327
2	301	125	151	205	279	417
3	210	102	95	196	318	340
4	265	108	151	170	218	415
5	286	128	113	193	297	428
6	216	107		216		
7	245			168		
8	221					
9	295					
10	230					
11	290					
Avg/4-day period	252 ± 12	114 ± 11	135 ± 29	190 ± 17	276 ± 38	386 ± 55
Avg/day	63	28	34	47	69	96
After hemoglobin infusions						
1	405	406	352	392	525	610
2	384	193	249	280	385	456
Total 8 days	789	599	601	672	910	1066
Avg/day	99	75	75	84	114	133
3	222	40	156	120	205	180
4	190	30	143	105	176	370
5		98	167	158	252	305
6		45		188	213	
7					202	
8					224	
Avg/4-day period	206 ± 22	53 ± 30	155 ± 12	143 ± 37	212 ± 25	285 ± 96
Avg/day	51	13	39	36	53	71

TABLE II. Increase in Fecal Urobilinogen Excretion Induced by Hemoglobin Infusions in 6 Patients.

Case No.	Avg urobilinogen excretion before infusion A 1 day mg	 8 days mg	Avg calculated hemoglobin converted to urobilinogen daily before infusion $\frac{A \times 28.5}{8 \times 10^3}$ g*	 %†	Amt of hemoglobin infused g	Urobilinogen excretion in first 8 days after infusion Total B mg	 Due to infusion B − A mg	Avg calculated infused hemoglobin converted to urobilinogen $\frac{(B-A)\,28.5}{10^3}$ g*	 %
1	63	504	1.79	29	8.84	789	285	8.12	92
2	28	228	.80	13	11.75	599	371	10.57	90
3	34	270	.97	16	7.80	601	331	9.43	121
4	47	380	1.34	21	9.76	672	292	8.32	85
5	69	552	1.97	32	11.25	910	358	10.20	91
6	96	772	2.74	44	8.30	1066	294	8.38	101
Grand avg	56	451	1.60	26	9.62	773	322	9.17	96

* Assuming that 1 mg of urobilinogen is derived from 28.5 mg hemoglobin.

† Assuming that 6.25 g of hemoglobin are destroyed daily.

in the urine. However, transient albuminuria without other urinary abnormality was noted in 3 of the patients. During infusions, plasma hemoglobin reached peak values of from 25 to 45 mg% and afterwards returned rapidly to base line. Plasma bilirubin, initially less than 0.4 mg%, became elevated to from 0.6 to 1.2 mg% after the infusions and returned within 1 to 4 days to its original level. No fever, fall of blood pressure or other adverse symptom developed in any patient.

Discussion. Abnormally increased erythrocyte destruction is accompanied by increased urobilinogen excretion attributed to this cause. However, in hematologically normal patients, despite large variations in daily urobilinogen excretion, on the average far less urobilinogen has usually been recovered than was expected. Thus, whereas estimated normal daily destruction of 6.25 g of hemoglobin should theoretically yield 219 mg of urobilinogen, Gilbertsen, working in Watson's laboratory, recently found an average daily excretion of only 140 mg, *i.e.*, a conversion efficiency of less than ⅔(1). This discrepancy is even more impressive in view of the evidence from normal subjects fed N^{15}-labeled glycine indicating that from 15 to 20% of the resulting N^{15}-labeled fecal stercobilin is not derived from the breakdown of the labeled hemoglobin of circulating red cells(6).

The control urobilinogen excretion values obtained in the present study account on the average for only 26% with a range of from 13 to 44% of the hemoglobin presumed to be catabolized daily. They are thus unusually low, perhaps because of the sluggish bowel habits of these inactive patients. In retrospect, the occasional enemas preliminary to certain stool collection periods were an ill-advised procedure. However, they could not have greatly lowered average values for urobilinogen excretion calculated from collections during at least 8 consecutive days thereafter. Moreover, the enema preceding the first post-infusion period collection would also have lowered its urobilinogen content. Nevertheless, following the hemoglobin infusions, the increase in amount of urobilinogen excretion accounted for an average of 96%, with a range in individual patients of from 85 to 121% of hemoglobin intravenously administered.

This striking increase over the control percentages suggests either that hemoglobin infused into the blood stream was more efficiently converted to bilirubin than when normally derived from effete red cells or that in the intestinal tract a more or less constant amount of potential urobilinogen fails to be excreted as such, irrespective of increased amount of bilirubin presented for conversion. Normally, except for the possible diversion by the kidney of a small amount of hemoglobin to a dipyrrylmethene such as pentdyopent, the evidence favors a quantitative conversion of hemoglobin to bilirubin and its quantitative excretion as bilirubin glucuronide by the liver(1). On the other hand, it is probable that the conversion of bilirubin to urobilinogen measurable by Ehrlich's aldehyde reagent is far from perfect. Thus, although fecal dipyrrylmethenes such as mesobilifuscin are now thought to be largely of anabolic origin(7), oxidative schism of tetrapyrryl bilirubinoid compounds to mesobilifuscin has been demonstrated *in vitro*(8) and this or other form of biochemical diversion might occur in the intestine or as an artifact of the fecal collections. Another possibility for explaining the reduced amount of urobilinogen excreted is that amount of urobilinogen normally reabsorbed from the colon and destroyed by the liver during the so-called enterohepatic circulation is greater than is generally supposed(9). Whatever the explanation, the present observations suggest that, when an increased amount of bilirubin is presented to the intestine, amount of bile pigment failing to appear as colorimetrically measurable fecal urobilinogen remains fairly constant so that relative proportion of bilirubin converted to urobilinogen increases. Thus, under normal circumstances the unknown diversion process must approach its maximum and so be incapable of much further augmentation.

Summary. In 6 adult patients without hematologic or hepatic abnormality the efficiency of conversion of bilirubin to colorimetrically measurable fecal urobilinogen, assuming a daily catabolism of 6.25 g of hemo-

globin, averaged only 26%. However, the efficiency of conversion of from 8.3 to 11.75 g of hemoglobin subsequently given by slow intravenous infusion averaged 96%. It is suggested that some physiological, biochemical or artifactual process normally diverts a more or less constant amount of bilirubin from conversion to fecal urobilinogen measurable by Ehrlich's aldehyde reagent in collected stools even when the bilirubin to be converted is significantly augmented.

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Received July 25, 1960. P.S.E.B.M., 1960, v105.

Effects of an Unsaturated and Saturated Lipid on Experimental Cholesterol Atherosis in Rabbits. (26078)

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Much interest has been shown in the relationship between the serum cholesterol and the iodine value of the dietary fat. It has been reported that feeding rabbits a highly unsaturated lipid in a diet that contains cholesterol increases serum cholesterol and amount of cholesterol atherosis(1,2). The purposes of this paper are: (1) to report some effects of adding an unsaturated and a saturated lipid with 2% cholesterol to the diet of rabbits; (2) to advance an explanation for the results.

When either a saturated or unsaturated lipid was fed to rabbits eating a diet containing cholesterol, higher values of serum cholesterol were produced. Increased amounts of cholesterol were also found in the aortas. Highest cholesterol values were observed in rabbits consuming the most unsaturated lipid. With addition of either lipid to the diet, fecal excretion of Lieberman Burchard chromogens and 3-β hydroxy sterols fell.

Method. Adult male rabbits were divided into 4 groups. Group I, the control group, was fed stock rabbit chow (Purina Rabbit Chow Checkers), Group II, the same stock diet with 2% cholesterol; Group III, stock diet with 2% cholesterol and 10% coconut oil, and Group IV, stock diet with 2% cholesterol and 10% linoleic acid (Fisher). The iodine number of the coconut oil fed was 9, and of the linoleic acid 130. These diets were continued for 8 weeks. Body weight and total serum cholesterol were determined at weekly intervals(3). During a 24 hour period of each week of experimental diets, fecal collections were made from the rabbits in Groups II, III, IV and Lieberman-Burchard chromogens, 3-β hydroxy sterols, and bile acids were measured(4). After 8 weeks the animals were killed and liver and aorta removed. The 3 cm of the aorta just distal to the aortic valve and a sample of liver were taken for cholesterol analysis. These tissues were homogenized, extracted with chloroform and total cholesterol estimated by the Kingsley and Shaffert Technic(5).

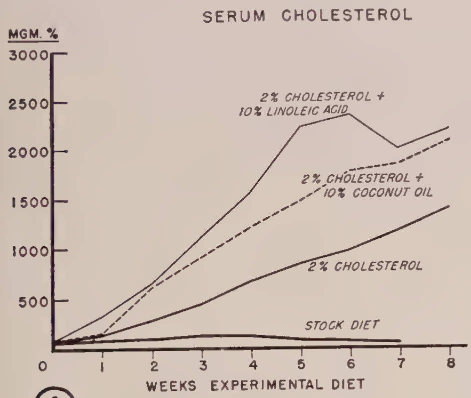
Results and discussion. The effect of the various diets on serum cholesterol is shown in Fig. 1. With addition of 2% cholesterol to stock diet the usual progressive rise in serum cholesterol was observed. With addition of either a saturated (coconut oil) or an unsaturated lipid (linoleic acid) to the diet con-

TABLE I. Effects of Unsaturated and Saturated Fats on Serum and Tissue Cholesterol.

Group		No. of animals	Final serum cholesterol, mg/100 ml	Aorta cholesterol —mg/100 g fresh tissue—	Liver cholesterol —mg/100 g fresh tissue—	% change TBW during exp. period, %
I	Controls	6	52 ± 6.2*	134 ± 16.7*	286 ± 27.9*	–11.0
II	2% cholesterol	12	1408 ± 138	211 ± 28.7	2280 ± 375	– 8.5
III	*Idem* + 10% coconut oil	12	2005 ± 226	253 ± 30.7	2190 ± 445	– 1.3
IV	" + linoleic acid	12	2390 ± 325	346 ± 64.2	6240 ± 875	–13.0

* ± S.E.

taining 2% cholesterol a further increase in average serum cholesterol was observed. Final average serum cholesterol for Group II was 1400 mg/100 ml; Group III, 2075 mg/100 ml; and in Group IV, 2200 mg/100 ml. It was concluded from these data that addition of a lipid to the diet containing cholesterol increased serum cholesterol whether the lipid was saturated or unsaturated.



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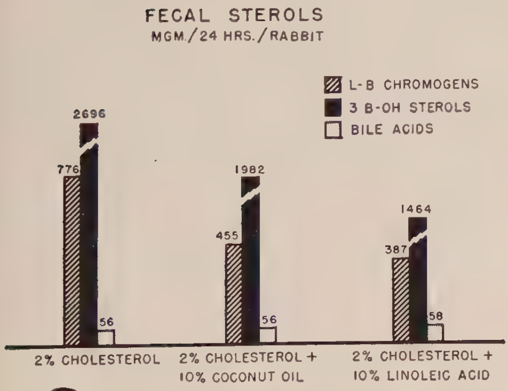

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FIG. 1. Effect of 2% cholesterol with added lipids on serum cholesterol of rabbits.

FIG. 2. Effect of various diets on excretion of certain fecal sterols.

Table I summarizes results of the tissue analyses for cholesterol. The highest aortic tissue cholesterol was observed in those animals that consumed 2% cholesterol with the most unsaturated lipid. The rabbits that ate the diet containing cholesterol and coconut oil had the next highest average aortic tissue cholesterol. It was therefore apparent that addition of a lipid to the diet of rabbits increased aortic tissue cholesterol above that observed when an equivalent amount of cholesterol was eaten without added lipid.

Liver tissue studies (Table I) revealed the liver of the rabbits on the stock diet to contain 286 mg cholesterol/100 g of fresh tissue. Addition of 2% cholesterol to the diet caused an 8 fold increase in liver cholesterol. When coconut oil was added to the diet no further increase in liver cholesterol was found but addition of linoleic acid further increased liver cholesterol so that 6% of total weight of the liver was cholesterol.

A possible explanation for the effect of the added lipid on serum and tissue cholesterol was found in studies on excretion of fecal sterols (Fig. 2). Animals eating the diet of 2% cholesterol excreted approximately 85% more Lieberman-Burchard chromogens than did animals eating the diets with added fat. Animals eating the diet containing 2% cholesterol excreted 56% more 3-β hydroxy sterols than did animals whose diet contained added fat. Despite these differences in excretion of cholesterol and 3-β OH sterols, bile acid excretion remained essentially the same in the 3 groups of animals studied. These results suggest that addition of either a saturated or an unsaturated lipid to the diet of the rabbit containing cholesterol decreases excretion of cholesterol. This probably means that addition of lipid to the diet increased absorption of the cholesterol and that a considerable portion of this cholesterol was retained. The fact that the animals eating the linoleic acid excreted the least amount of 3-β hydroxy sterols and Lieberman-Burchard chromogens and had the highest concentration of cholesterol in the liver suggests that linoleic acid was the more effective of the two lipids in promoting cholesterol absorption.

Summary. Adding fat to the diet of the rabbit which contains cholesterol increased serum cholesterol. The increased serum cholesterol was associated with increased aortic tissue cholesterol. It is proposed that addition of the lipid to the diet increased absorption and retention of cholesterol and this intensified the cholesterol atherosis.

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Received July 28, 1960. P.S.E.B.M., 1960, v105.

In vivo Tumor Inhibitory Activity of Normal Human Serum.* (26079)

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During the past few years increasing interest has been focused on the effect of serum and its components on normal and malignant animal cells. Willheim *et al.*(1,2) have shown that normal human serum is able to digest Ehrlich ascites and other tumor cells. Serum obtained from schizophrenic patients was shown by Federoff(3) to be toxic to strain L cells. Bolande and co-workers(4,5), have demonstrated cytotoxic action of serum for HeLa cells, U-12 cells of human origin, Ehrlich ascites and Sarcoma 180 cells. Björklund(6) has presented evidence for presence of a cytotoxic factor in normal serum coupled to an inhibitor capable of rapid and complete destruction of human tumors *in vitro*. The studies presented here, initiated 3 years ago, deal with comparisons of *in vivo* tumor inhibitory activity of sera from normal individuals and sera from patients with diseased states on Sarcoma 180 cells. It was found that normal human serum exerted a greater inhibitory effect on Sarcoma 180 ascites tumor growth than sera from patients with leukemia and related diseases. Attempts were also made at partial isolation and characterization of the normal human serum components toxic to the tumor cells.

Materials and methods. Donor CFW mice with Sarcoma 180 tumor in the ascitic form were sacrificed under ether anaesthesia, 6-8 ml of non-hemorrhagic peritoneal fluid was withdrawn and immediately placed in 1.0 ml saline and 0.2 ml heparin (1000 USP units/ml). A cell count was made on an aliquot of the tumor suspension, and the remainder diluted with cold pH 7.4 isotonic saline so that each inoculum of 1 ml contained 2 x 10^6 cells.

Blood was withdrawn from healthy subjects and from patients prior to initiation of therapy. Rabbit, guinea pig, mouse and rat blood were obtained by cardiac puncture; and bovine, porcine, calf and lamb blood collected from the jugular vein. Defibrination was immediately carried out by agitating blood with glass beads and the cellular elements centrifuged at 3000 g in the cold. The supernatant was aspirated and frozen at −70°C. All subsequent handling of the sera and their fractions was carried out rapidly between 0 and 3°C.

A 90% saturated ammonium sulfate solution of normal human serum (NHS) was prepared by addition of solid ammonium sulfate,

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* This investigation was supported by a research grant from NIH, USPHS.

TABLE I. Effect of Various Sera and Sera Fractions on Survival of Mice Inoculated with Sarcoma 180 Cells.

Inoculum	No. of sera tested	No. of animals tested	% mice surviving	
			17 days	120 days
Tumor + isotonic saline	—	260	0	0
" + normal human serum	13	250	79 ± 12*	25 ± 13
" + 90% $(NH_4)_2SO_4$ precipitate of normal human serum	10	150	58 ± 13	25 ± 10
" + rabbit serum	3	45	16 ± 15	0
" + guinea pig serum	2	24	0	0
" + mouse "	1	15	0	0
" + rat "	2	32	0	0
" + bovine "	3	50	8 ± 10	0
" + porcine "	2	48	8 ± 8	0
" + calf "	2	48	8 ± 8	0
" + lamb "	2	50	4 ± 4	0
" + schizophrenic serum	4	60	88 ± 19	27 ± 15
" + serum from patients with blood dyscrasias	8	120	28 ± 14	0

* Mean ± S.D.

over a period of 10 minutes, to gently stirred serum. pH of the mixture was maintained between 6.4 and 7.5 with N NH_4OH throughout addition of the solid. After additional 5 minutes of stirring the resultant precipitate was centrifuged for 30 minutes at 10,000 g. The precipitate was dissolved and made up to its original serum volume with pH 7.4 isotonic saline and dialyzed for 18 hours against 20 times its volume of isotonic saline.

Swiss mice weighing 20-25 g each were given inoculations intraperitoneally, first of 1 ml tumor suspension containing 2 x 10^6 cells, followed directly with 2 ml of cold pH 7.4 serum or its fraction. A control group with each mouse receiving tumor and isotonic saline was run concurrently with each experiment. Groups of 15-20 mice were used for each test run and a similar number were used as a control. Gain in weight and length of survival were taken daily as an index of the amount of ascites formed and degree of malignancy induced. All dead mice were autopsied for presence of tumor cells and only those mice that had tumor were scored as tumor deaths.

Results. All control mice died in less than 17 days after injection of tumor cells and saline, whereas animals that received tumor cells and sera from healthy subjects were well protected at 17 days and one quarter of the original population lived for more than 120 days (Table I). The property of NHS for inhibiting tumor growth was found to be heat-labile in that when NHS was heated to 60° for 30 minutes all animals receiving this solution and tumor cells developed tumors. Most were dead at 17 days and none survived more than 120 days. Furthermore, tumor inhibitory activity of NHS was found to be non-dialyzable, precipitated in the euglobulin fraction, and its activity was found to be independent of Ca^{++} and Mg^{++}. In addition, between the pH range tested, 5-9, serum was found to exert its tumor inhibitory action. Finally, in contrast to NHS, sera of rabbit, guinea pig, mouse, rat, bovine, porcine, calf and lamb failed to protect mice against tumor development (Table I).

The precipitate obtained from NHS saturated with 90% ammonium sulfate gave mice a degree of protection against tumor about equivalent to that of whole NHS (Table I). However when 0-40, 40-50, 50-60. 60-70, and 70-90% saturated ammonium sulfate subfractions were prepared, in most experiments, a low tumor inhibitory activity was recovered in these fractions.

The abnormal sera group consisted of clinically diagnosed but untreated cases of schizophrenia and patients with various malignant diseases. The latter group was composed of 2 patients with acute leukemia, 2 with chronic myeloid leukemia, one with polycythemia vera and 3 with chronic lymphocytic leukemia. The results (Table I) indicate

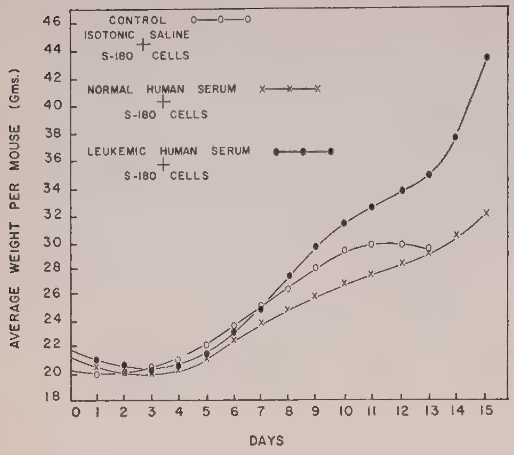

FIG. 1. Effect of saline, normal human serum and leukemic serum on growth of Sarcoma 180 ascites tumor cells in Swiss mice. Inoculation of a group of 15-20 mice with 2 × 10^6 Sarcoma 180 ascites tumor cells and 2 ml saline/mouse (control) resulted in rapid growth of tumor and death of all animals in 13 days. Substituting 2 ml normal human serum for saline resulted in marked inhibition of tumor development. No such inhibition was, however, observed when leukemic serum was used.

that schizophrenic serum, like NHS, also possesses *in vivo* tumor inhibitory activity. When mice, on the other hand, received an inoculum of Sarcoma 180 tumor cells and serum from patients with blood dyscrasias, the percent of animals surviving 17 days averaged lower than those given tumor cells and NHS. Furthermore, none of the mice that received tumor cells and serum from patients with malignant diseases survived for more than 120 days. Rate of tumor growth in mice receiving the latter serum was more rapid than in animals given NHS (Fig. 1).

Discussion. Although the advantages of an *in vitro* test system are numerous, the final test of cytotoxic activity of an agent must be elicited in an *in vivo* system. The present studies show, indeed, that NHS contains non-specific components inhibitory to Sarcoma 180 proliferation *in vivo*, whereas such activity appears to be low or lacking in serum of individuals with various blood dyscrasias.

It is a common clinical observation that patients with leukemia receiving blood transfusions exhibit a transient fall in leukocyte count as well as a general improvement in well being. That NHS may contain certain factors capable of destroying atypical cells *in vivo* is also suggested by Southam and Pillemer(7) who found that normal subjects rejected cancer cell implants whereas in patients with advanced carcinoma, growth of the implanted cells occurred at almost all sites.

The tumor inhibitory activity of NHS described here does not appear to be properdin (8) since no Mg^{++} or other divalent cations were required for such activity. Furthermore, the serum of animals other than man, known also to contain properdin, failed to inhibit tumor growth *in vivo*. It is possible that the components of NHS described herein may, however, be similar or related to the cytotoxins of Bolande and co-workers(4,5).

In vivo tumor inhibitory activity was also shown to be present in schizophrenic serum (SS). It is at present unresolved whether the activity of SS is due to the same component or components of NHS, or if SS contains additional *in vivo* tumor inhibitory factors. Federoff(3) using mouse strain A cells in tissue culture found that both NHS and SS were toxic to these cells. However, degree of toxicity was found to be greater for blood sera of schizophrenics than for sera of healthy individuals.

Summary. Normal human serum contains a heat-labile, non-dialyzable, ammonium-sulfate precipitable euglobulin fraction that inhibited growth of Sarcoma 180 cells *in vivo*. This component was found to be active between pH 5-9 and required no divalent cations. Schizophrenic serum was also found to be inhibitory to Sarcoma 180 cells. Sera of individuals with blood dyscrasias failed to protect mice against tumor development.

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Received July 28, 1960. P.S.E.B.M., 1960, v105.

Insulin Potentiating Action of Tolbutamide in Eviscerated Cats. (26080)

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An increase in glucose uptake of hepatectomized rats, rabbits and dogs has been produced by injection of tolbutamide(1,8,9). The author has shown the same in hepatectomized cats(5). He explained this effect as probably due to a "potentiation" of insulin secreted in the animals. This explanation is supported by the present experiments.

Methods. Nembutal anaesthetized cats were eviscerated and nephrectomized. Glucose was then infused *i.v.* at a constant rate sufficient to keep the blood sugar level nearly constant. Groups of 6-7 animals were treated as follows: Two groups were injected with insulin 0.01 unit/kg *i.v.* One of these received tolbutamide 100 mg/kg *i.v.* 5 minutes before insulin injection. A third group received only tolbutamide 100 mg/kg *i.v.* The fourth group served as controls, which were injected with a volume of 0.9% saline similar to that of the tolbutamide injection in the 2 preceding groups. Blood sugar level was followed at 10 to 20 minute intervals for 2 hours before and 3 hours after injections. Blood sugar determinations were made by the Somogyi-Nelson technic(7) on heparinised plasma obtained from arterial blood samples immediately after bleeding.

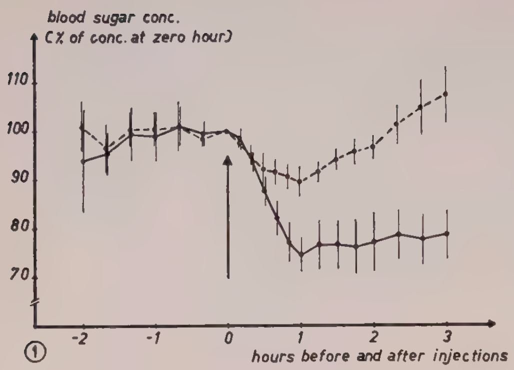
Results. The results are shown in Fig. 1 and 2 giving average values of the 4 groups, blood sugar concentrations being expressed as percentages of the level at zero hour. Absolute blood sugar values at this time varied between 82 and 160 mg %.

Fig. 1 demonstrates how the hypoglycemic effect of insulin (0.01 unit/kg) is reinforced by tolbutamide. T-tests shows that corresponding points in the 2 curves differ significantly 40 minutes after the medication ($P < 0.05$). After 60 minutes P was less than 0.01. It kept this value during the rest of the experiment except 75 and 120 minutes after the injections, where $0.01 < P < 0.05$.

Fig. 2 shows a slight effect of tolbutamide given without insulin to eviscerated, nephrectomized cats. The difference between the curves is not statistically significant at the 5% probability level. The blood sugar rises slightly in the last half of the experimental period when only saline is injected, while it remains practically unaltered during the 5 hours of the experiments when tolbutamide is given.

Comparing corresponding differences in the pair of curves in Fig. 1 and 2 it is found that the hypoglycemic effect of tolbutamide is 2½-6 times as pronounced when given with insulin as when given alone. This clearly indicates an insulin "potentiating" effect, although the difference between the tolbutamide effects in Fig. 1 and 2 can not be proved statistically at the 5% probability level with the present number of experiments. The insulin potentiation appears partly as a more pronounced, partly as a more sustained hypoglycemia than when insulin is given alone.

Discussion. The mechanism of this insulin potentiation is obscure, but the maintained hypoglycemia could be explained by an inhibited insulin degradation, as proposed by Mirsky(6). If this is the case, the feeble effect seen in eviscerated animals not given insulin (Fig. 2) might be due to preservation of small amounts of insulin remaining in the animals from before the evisceration. This possibility is at present under investigation



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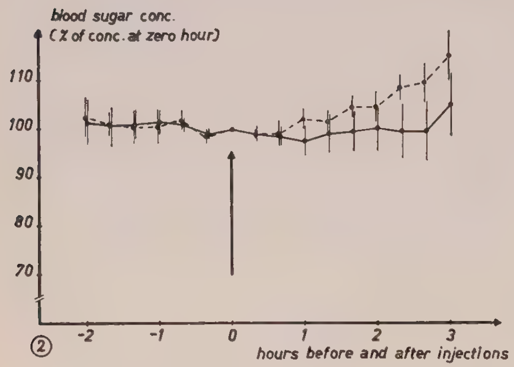

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FIG. 1. Blood sugar response of eviscerated cats to insulin 0.01 unit/kg i.v. (dotted curve—avg of 7 animals) and to tolbutamide 100 mg/kg + insulin 0.01 unit/kg i.v. (solid curve—avg of 6 animals). Injections at the arrow. S.E. of mean indicated by vertical lines.

FIG. 2. Course of blood sugar conc. after inj. of saline (dotted curve—avg of 7 animals) and tolbutamide 100 mg/kg i.v. (solid curve—avg of 7 animals). Injections at the arrow. S.E. of mean indicated by vertical lines.

on cats pancreatectomized 2 days before evisceration.

Several investigators(2,3,4,10) including the author(5) have reported that tolbutamide is without effect in eviscerated animals. In some of these works, including the author's, lack of control experiments may have concealed a possible effect of the tolbutamide, as the constant glucose uptake in eviscerated animals injected with tolbutamide gives the impression that tolbutamide has no effect.

The results reported here are in accordance with the findings by Houssay *et al.*(3) of a hypoglycemic effect of tolbutamide in eviscerated dogs continually infused with glucose and insulin.

Summary. An insulin potentiating action of tolbutamide in eviscerated, nephrectomized cats is reported.

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Received August 4, 1960. P.S.E.B.M., 1960, v105.

Delayed Monoarthritis in Rabbits Induced by Means of Avian Nephrotoxic Serum.* (26081)

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In a series of experiments in which Duck Anti-Rabbit Kidney Serum (DARKS) was injected into rabbits by various routes other than intravenous, the specific affinity of the serum for kidney tissue appeared doubtful. Based on a pathogenetic concept suggested by these experiments, small amounts of DARKS were injected repeatedly into the stifle of rabbits in the attempt to provoke an arthritis of immunogenic origin. This study presents preliminary experiments in which animals thus treated developed a delayed monoarthritis at the site of injected joint.

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* This work was supported by grant from Nat. Inst. of Health.

Materials and methods. The study was carried out on 14 New Zealand white rabbits of either sex, weighing between 3 and 4 kg, divided into 4 groups.

1) Four animals received Duck Anti-Rabbit Kidney Serum (DARKS) in the right stifle (knee joint). 2) Four other animals received DARKS in the right stifle and Normal Duck Serum (NDS) in the left. 3) Two rabbits received NDS in the right stifle. 4) Four animals were pre-immunized with 2 injections of 0.1 and 0.4 ml NDS given intradermally at 2 day intervals. Six months later one of them received 0.3 ml DARKS, another 0.3 ml NDS, in the right stifle. The remaining 2 animals were treated similarly except that the intra-articular injection was given after a delay of another 3 months.

Intra-articular injections with either NDS or DARKS were given through the anterior interline of the knee joint twice weekly for 6 weeks in amounts of 0.1 and 0.3 ml for the first week and 0.5 ml for the remaining 5 weeks.

DARKS was obtained from ducks immunized with a 10% suspension of blood-free, homogenated rabbit kidneys following the procedure previously described(1). NDS was obtained from normal duck blood submitted to the same processing routine as the DARKS. All rabbits were periodically bled. The serum was frozen and stored. Renal damage was ruled out by urinalysis, by routine blood tests previously described(1) and by histologic examination of the kidneys.

Careful clinical and roentgenologic evaluations of the knee joints were periodically performed on all animals. The severity of the arthritis during the acute stage was expressed as 1+ to 5+ the highest number indicating severe clinical symptoms.

Antibody titers of the various samples of rabbit serum were determined semi-quantitatively by the precipitin reaction between rabbit and duck sera in capillary tubes incubated at 37°C for 4 hours and placed afterwards into the refrigerator overnight. Intensity of the reaction was measured by the height of the precipitated columns and expressed as 1+ to 5+. A precipitin reaction filling approximately 50% of the capillary tube was estimated as 5+.

The rabbits were sacrificed by overdosage with barbiturates. The kidneys and both knee joints were immediately fixed in 10% buffered formalin solution for histologic studies. After decalcification and paraffin embedding, sections were stained with hematoxylin and eosin and a modification of the Masson technic(2).

Results. Four of the 8 rabbits which had no previous experience with duck serum, developed a severe monoarthritis during the 6 week period following the last intra-articular injection of DARKS. The remaining 4 animals also developed arthritis but only 2 weeks after a "booster" injection of 0.5 cc DARKS. This injection was given intra-articularly, 9 weeks after termination of initial treatment.

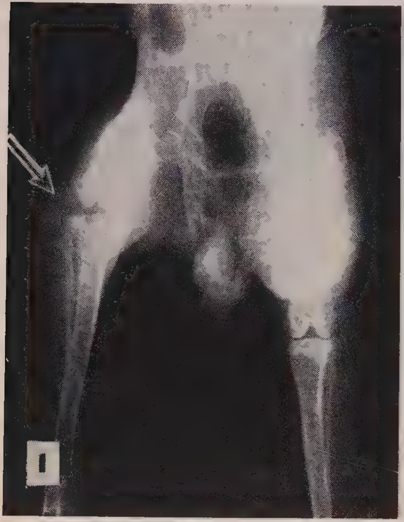
Animals injected into each stifle, *i.e.*, DARKS into the right and NDS into the left, developed a monoarthritis only in the stifle injected with DARKS (Fig. 1).

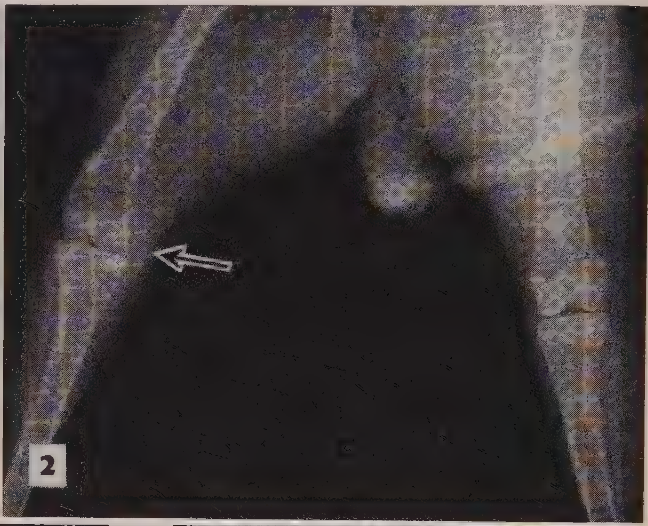
The 4 animals which had been injected with NDS intradermally several months previously, developed an *accelerated* arthritis within one week following the first intra-articular injection of either DARKS or NDS.

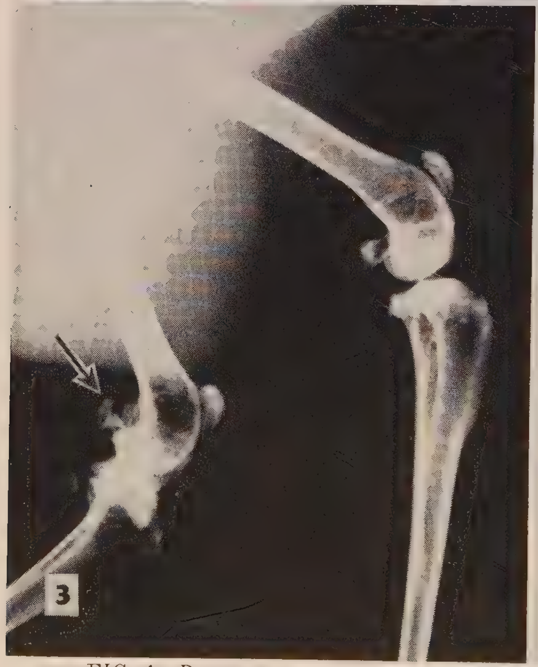
The first clinical sign of arthritis was swelling. It was obviously associated with pain and marked functional impairment as revealed by the reactions and posture of the animals. Crepitus and limitation of passive luxation of the rotula were the most frequent additional findings. The acute stage was usually followed by a chronic, progressive evolution in which hyperostosis, deformation of the condyles and functional limitations were the main clinical features. Serial roentgenographic studies taken during development of arthritis showed 3 main stages of evolution: a) Swelling of the soft tissues with bulging of joint capsule (Fig. 2). b) disappearance of the cartilage with narrowing of condyles and hyperostosis (Fig. 3), eventually leading to c) osteosclerosis (Fig. 4).

The gross pathologic findings varied with the stage of arthritis. Dullness of the cartilage, bone lipping at periphery of the cartilage, thickening of the capsule, hyperostosis with new bone formation in the soft tissues were the common gross autopsy findings.

Microscopic examination of the decalcified sections of joints showed diffuse infiltration of the synovia and cartilage by segmented leukocytes and ulceration of the cartilage leading to exposure of the underlying bone.







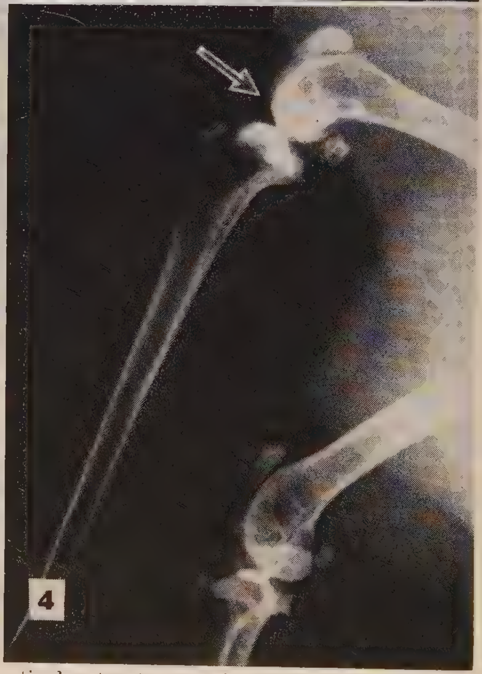


FIG. 1. Roentgenogram taken 4 wk after intra-articular treatment with DARKS, (right stifle), and NDS, (left stifle). Arrow points the severe arthritis of the joint receiving DARKS. The left stifle treated with NDS appears unchanged.

FIG. 2. Roentgenogram taken 6 wk after last intra-articular inj. with DARKS. There is bulging of joint capsule and swelling of soft tissues (arrow) representing first stage in development of arthritis.

FIG. 3. Same animal as in Fig. 2, 12 wk later. Arrow points at hyperostosis as second stage of arthritis.

FIG. 4. Same animal as in Fig. 3, 4 wk later. Arrow points at osteosclerosis as final stage.

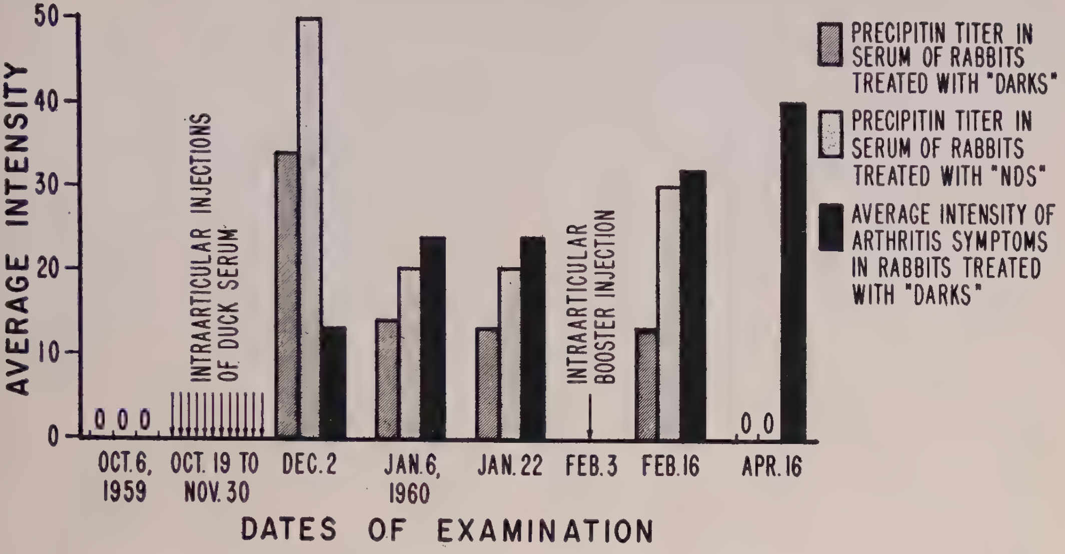


FIG. 5. Avg anti-duck precipitin titer in blood of rabbits treated with either DARKS or NDS given intra-articularly twice weekly for 6 wk. Avg intensity of arthritic symptoms increases as serum precipitin titer decreases in rabbits treated with DARKS. Avg intensity of arthritic symptoms as well as of precipitin reactions are computed according to the conventional formula:

$$\text{Avg intensity} = \frac{\Sigma\,(n +) \times 10}{N}$$

$\Sigma\,(n +)$ = sum of (+) expressing severity of symptoms or intensity of precipitin reaction for individual animals of each group. N = No. of animals in each group.

The joint capsule was edematous, congested and also infiltrated with polymorphonuclear leukocytes. The uninjected contralateral joint which served as control appeared normal. The joints which received NDS also failed to develop arthritic changes within 6 months despite intra-articular booster injections. The accelerated arthritis of the pre-immunized animals did not follow the evolutionary pattern of the delayed arthritis but showed a tendency for recovery after the acute period.

The serum of rabbits which had no previous experience with duck serum showed no precipitin reaction with the latter before intra-articular injection. At the end of intra-articular treatment, *i.e.,* shortly before development of arthritis, all serum samples showed intensive precipitin reaction with either DARKS or NDS. In the animals treated with DARKS the precipitin titer diminished progressively and finally disappeared as the arthritis developed (Fig. 5). Control animals injected with NDS only did not develop arthritis and maintained the anti-duck precipitin titer in serum at higher levels and for a longer period than those receiving DARKS (Fig. 5). A direct relationship seems to exist between development of arthritis and disappearance of specific antibodies from the circulating blood.

Discussion. These experiments strongly suggest that the monoarthritis produced by DARKS is of immuno-allergic nature. This is supported by several facts: 1) Arthritis is delayed by at least 8 weeks after the first, and 2 weeks after the last intra-articular injection. 2) It is accelerated when intra-articular treatment is performed on animals pre-immunized with duck serum. 3) Development of arthritis coincides with a sudden diminution or disappearance of the circulating anti-duck antibodies, suggesting their exhaustion at site of affected joint.

A superimposed infection as a cause of arthritis at the DARKS-treated joint, although possible, is not probable. All batches of nephrotoxic serum produced arthritis, while normal duck sera given simultaneously into the contralateral joint produced no changes. The long delay between intra-articular treatment and development of arthritis also tends to eliminate the possibility of superinfection as main or contributory factor. Furthermore, PPLO have been ruled out by repeated negative cultures.

Despite the fact that both DARKS and NDS produced similar titers in the serum of the host rabbit, only the former was able to produce joint alterations when given intra-articularly, skin necrosis when given intradermally, and kidney lesions when given intravenously. This suggests that DARKS contains antibodies reacting with an element, possibly of mesenchymal origin, common to the 3 different organs, joint, skin and kidney. According to Izumi(3) and Chikamitsu(4), the active principle of the nephrotoxic sera responsible for the damage to the skin as well as to the kidneys are antibodies against mesenchymal elements of the vascular system. The elective, almost exclusive, involvment of the renal vascular system when nephrotoxic serum is given intravenously is due, according to Izumi, to the specific functional and structural character of the kidney which, besides having one of the richest vascular supplies in the body, is capable of concentrating, by ultrafiltration, the various components of the circulating blood(3). Chikamitsu also claims that nephrotoxic sera do not contain antibodies against kidney tissue but against the vascular system in general. His conclusion is based on the fact that when duck anti-rabbit pneumotoxic serum is injected intravenously into rabbits, the animals do not develop pulmonary damage but a pure Masugi nephritis(4). The anti-lung or pneumotoxic serum is, allegedly, a pure anti-vascular serum because of the rich capillary system of the lung.

Studies are in progress, using the fluorescent antibody technic, as to whether the arthritis provoked by the nephrotoxic serum in rabbits is initiated by vascular damage or whether the duck protein becomes attached to other cellular elements of the joint.

Summary. 1. Normal rabbits injected with 0.5 ml of duck anti-rabbit kidney serum (DARKS) in the stifle, twice weekly for 6 weeks, developed a *delayed* monoarthritis starting 2 to 4 months following first intra-articular injection. 2. Control animals injected with a similar dose of normal duck serum (NDS) failed to develop arthritis within a 6 months observation period. 3. The intra-articular injections of either NDS or DARKS produced high serum antibody titers precipitating both, NDS and DARKS prior to development of the arthritis. The titer disappeared as the arthritis developed in rabbits treated with DARKS but persisted in the NDS-treated control animals. 4. When either, DARKS or NDS, was injected into the joint of rabbits previously immunized with NDS, an accelerated arthritis developed within one week. 5. The main clinical symptoms at site of the affected joint consisted of pain, swelling, crepitus, limitation of active and passive movements with subsequent functional impairment. 6. Histologic studies revealed exudate in the joint cavity consisting predominantly of PMN's and infiltration of synovia and cartilage with segmented leukocytes as well as erosion of the cartilage leading to exposure of the underlying bone. 7. The clinico-pathologic findings were paralleled roentgenologically by swelling of the soft tissues, bulging of the articular capsule, hyperostosis, disappearance of the cartilage, narrowing of the condyles, resulting in osteosclerosis at the end of 6 months.

The authors wish to acknowledge valuable contribution of Dr. Alexander Lewitan to the roentgenologic study presented in this paper.

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Received August 2, 1960. P.S.E.B.M., 1960, v105.

Nine Year Summary of Parthenogenesis in Turkeys.* (26082)

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Experiments at this station during the past 9 years have demonstrated, repeatedly, that unfertilized eggs of turkeys can develop to varying degrees upon being incubated. The original observations were made in 1952. Subsequent yearly tests of eggs from selected virgin turkey females have fully confirmed earlier observations. Some of these data have been published(1,2,3,4); but the major portion has not; hence it is desirable that a complete summary be presented.

Confirmation of the occurrence of parthenogenesis in turkey eggs has come from other laboratories. At Oregon State College, Parker noted in 1958 an unorganized type of growth in unfertilized Beltsville Small White turkey eggs following a 14-day period of incubation (personal communication). Kosin, Sato and Nagra, at State College of Washington observed parthenogenetic development in unfertilized eggs from Broad Breasted Bronze, Washington State White, and Beltsville Small White turkeys. These investigators reported on 46 embryos, including one surviving to time of hatch, at the 11th World's Poultry Congress, Mexico City, 1958. Cytological observations based on parthenogenetic tissues were published by Kosin and Nagra(5), Sato and Kosin(6), and Kosin and Sato(7). Their studies, as well as our own(8,9), established diploidy in the somatic cells of parthenogenetic tissues.

Material and methods. Each year, certain Beltsville Small White females which as virgins had demonstrated their ability to produce eggs showing a high incidence of parthenogenesis, were used as breeders. These selected females were mated to males from families in which the daughters also had demonstrated their ability to produce eggs exhibiting a high incidence of parthenogenesis.

The poults hatched for replacement stock each year were pedigree banded, brooded in batteries until 6 weeks of age, and then transferred to a brooder house. At ages varying from 4-12 weeks, they were separated from their immature male pen mates. Sex identification was accomplished by performing a laparotomy and observing the gonads. From the time of sex identification until maturity, the virgin females were reared in wire enclosed pens to insure against any possible contact with sexually mature males. At the age of 30-36 weeks, the females were transferred to laying houses. Some birds were placed in unheated pens within a large turkey house, others in individual wire cages within a heated building. All birds received the same standard laying mash. Electric lights, used during early morning hours to supplement natural daylight, maintained a 14 hour photoperiod.

Eggs were gathered from trapnests and wire cages, 3 to 4 times daily, and as collected were placed in an incubator at a temperature of 99.5°F and a relative humidity of 57%. In 1952, the eggs tested were given an initial 7-day period of incubation before being candled but in later tests this period of incubation was lengthened to 9-10 days. Eggs in which live embryos could be detected by candling were replaced in the incubator. Those eggs showing growth of membranes only, or no development, were removed, broken, and the discs of each reexamined. Only embryonic growth that could be detected macroscopically was listed as parthenogenetic. All questionable cases and eggs showing no decernible development of the disc were classed as non-parthenogenetic.

Eggs showing parthenogenetic development were classified into 3 categories; (1) those in which the development consisted solely of

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* The author wishes to express his thanks to Dr. Richard Fraps, U. S. Dept. of Agric., and to Dr. Mary Juhn, Univ. of Maryland, for critically reviewing this manuscript and for their interest in this project since it was initiated.

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TABLE I. Incidence of Parthenogenetic Development in Unfertilized Eggs of Beltsville Small White Turkeys.

Year	No. of hens	No. eggs tested	No. and % of eggs found to contain: Membranes only		Blood and membranes		Embryos		All categories of parthenogenesis	
			No.	%*	No.	%	No.	%	No.	%
1952	29	934	156	16.7	0		2	.2	158	16.9
1953	23	1463	191	13.1	12	.8	3	.2	206	14.1
1954	103	5930	1198	20.2	71	1.2	44	.74	1313	22.1
1955	110	5757	1251	21.7	161	2.8	84	1.4	1496	26.0
1956	128	6003	1381	23.0	198	3.3	251	4.2	1830	30.5
1957	181	8608	2293	26.6	490	5.7	360	4.2	3143	36.5
1958	214	8045	2060	25.6	492	6.1	722	9.0	3274	40.7
1959	78	2926	718	24.5	153	5.3	342	11.7	1213	41.5
1960	60	2815	720	25.6	123	4.4	277	9.8	1120	39.8
Totals & avg	926	42,481	9968	23.5	1700	4.0	2084	4.9	13,752	32.4

* Percentages are on basis of total eggs tested.

growth of the extraembryonic membranes; (2) those in which differentiation had proceeded to the extent that blood islands or blood vessels were clearly visible by candling or by macroscopic examination and (3) those in which embryos were identifiable on gross examination. Embryos were further classified as to age in terms of equivalent development of the normal turkey embryo. Number of embryos surviving to hatching and those helped from the shell were also listed.

Results and discussion. Results based on examination of more than 42,000 eggs from virgin Beltsville Small White turkeys are summarized in Table I. These data show a steady increase in overall incidence of parthenogenesis, with the most marked increases coming in the categories "blood and memranes" and "embryos." In 1953, 0.8% of all eggs examined were classified in the category of "blood and membranes," while in 1958 6.1% were so classified, representing a 7 fold increase. In 1952, embryos were found in 0.2% of all eggs tested, whereas in 1958 the proportion was 11.7%, a 57-fold increase over that observed in eggs of unselected hens used as foundation stock. All embryos and poults in which sex has been determined either by gross(3) or by cytological(10) examination have been male.

Data presented in Table II give number of embryos and poults encountered each year. Also shown are numbers and percentages of embryos that attained the size of normal 2-10, 11-20, and 21-28-day turkey embryos. Listed separately are numbers of parthenogenetic embryos surviving to hatching; most of these were helped from the shell. A definite increase in average age attained by parthenogenetic embryos since 1952 is clearly seen. The increased embryonic viability is

TABLE II. Number and Percentage of Parthenogenetic Embryos Encountered Each Year and Size Each Attained in Relation to That of a Normal Turkey Embryo.

Year	Total No. embryos	Stage of development (days): 2-10		11-20		21-28		Hatched	
		No.	%	No.	%	No.	%	No.	%
1952	2	2	100.0						
1953	3	3	100.0						
1954	44	37	84.1	2	4.5	4	9.0	1	2.3
1955	83	70	84.3	5	6.1	8	9.6	0	.0
1956	252	166	65.9	19	7.5	59	23.4	8	3.2
1957	360	249	69.2	28	7.8	71	19.7	12	3.3
1958	721	492	68.3	75	10.4	134	18.5	20	2.8
1959	342	209	61.1	36	10.5	85	24.9	12	3.5
1960	277	155	56.0	20	7.2	87	31.4	15	5.4
Totals & avg	2084	1384	66.4	185	8.9	448	21.5	68	3.3

especially pronounced in the relative number of embryos surviving for 11 days or longer. For example, in 1954 4 of 44 or 9.0% of the embryos attained a size equivalent to that of 21-28-day normal turkey embryos, whereas in 1960 87 of 277 embryos or 31.4% were listed in this age category. Increased viability of parthenogenetic embryos is also reflected in the number of embryos completing their incubation period. Since 1956, 67 poults have been hatched, many of which lived for some time, a few surviving to maturity. Viable spermatozoa have been observed in semen samples obtained from each of 3 parthenogenetic males and offspring have been sired by one male, Olsen(11). In 1960, 15 poults were hatched, 4 of which are still alive, ranging in age from 71 to 136 days. These evidences of increased embryonic viability can be attributed in large part to the selective breeding program initiated in 1954.

Summary. A breeding program for increasing parthenogenetic development, based on the progeny test method, was initiated in 1954. More than 42,000 eggs from 926 Beltsville Small White virgin turkey hens were involved in this 9-year study. A marked increase in incidence of parthenogenesis, particularly of the more highly organized type, has resulted. Parthenogenetic development was observed in 16.7% of the eggs tested in 1952, in 41.7% in 1959. In 1952, embryos were encountered in 0.2% of all eggs whereas in 1959 11.7% of the eggs were so classified. Of 44 embryos found in 1954, 4 or 9% survived until 21-28 days of incubation; in 1960, 87 of 277 or 31.4% did so. Since 1956 a total of 67 embryos, all males, have survived to hatching, a few reaching maturity. Three males produced spermatozoa and one sired offspring.

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Received August 1, 1960. P.S.E.B.M., 1960, v105.

Suppression of Human Choriocarcinoma Maintained in the Hamster Cheek-Pouch by Extracts and Alkaloids of *Vinca rosea*. (26083)

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Noble, Beer and Cutts have reported their pioneer studies on the chemical and biological properties of crude extracts of *Vinca rosea*,* and of the alkaloid, Vincaleukoblastine, initially isolated by them from these crude plant extracts(1,2). They noted the marked marrow depressant action of these preparations in rats and described an interference with estrogen-induced uterine growth in the immature rat as well as carcinostatic effects produced by certain of the active plant fractions. The marrow-depressant, carcinostatic, and uterus-inhibiting properties appeared to resemble the known biological effects of such folic acid antagonists as Methotrexate (4-amino - N10 - methyl - pteroylglutamic acid). Since we had previously observed marked regression of choriocarcinoma in women following intensive Methotrexate therapy it seemed

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* The extracts of *Vinca rosea* as well as the specific alkaloids derived therefrom were kindly furnished by Dr. Irving Johnson and Dr. G. H. Svoboda, Eli Lilly Co. Research Labs., Indianapolis, Ind.

TABLE I. Inhibitory Effect of Extracts and Alkaloids of *Vinca rosea*.*

Preparation	Dose, mg/kg†	% inhibition‡
Crude extract	11.25	30
	22.50	50
	33.75	90
	30.0	90
Vincaleukoblastine	.75	90
	.375	40
	.75	80
	.75	90
	.75	90
	.75	80
	.375	50
Leurosine	75.0	90
	37.5	90
	18.75	60
	9.37	40
	4.68	0
Virosene	3.75	0
Lochnerine	3.75	0
Perivine	3.75	0
Catharanthine	75.0	0
	150.0	0
Vindoline	75.0	0
	150.0	0

* WO strain of Erwin-Turner choriocarcinoma used throughout.

† Dosages indicated are daily subcut. inj. started on day 7 after transplantation and continued for 6 days; 8 to 10 animals/group.

‡ % inhibition estimated from comparison with saline treated controls.

desirable to determine the potential inhibitory effect of extracts of *Vinca rosea* and of its alkaloids(3,4) upon human choriocarcinoma maintained in heterologous transfer in the hamster cheek-pouch(5). We were further encouraged to proceed with these studies by the observations of Johnson concerning the oncolytic effect of these preparations in a number of animal tumors(6).

Materials and methods. In these studies we have employed the WO strain of the Erwin-Turner choriocarcinoma maintained in the untreated female hamster as previously described(5). The crude *Vinca* extracts and the respective alkaloids were prepared and characterized by methods described by Svoboda *et al.* and by Gorman *et al.*(3,4). The drug was started on the 6th day after transplantation and after initial growth of the transplant could be clearly observed. The materials were given subcutaneously in the dosage regimen detailed in Table I. The growth pattern of the transplanted tumor was determined by direct inspection and recorded by free-hand, life size sketches.

Such records are admittedly suitable for recording only very substantial differences in growth rate. However, they permit an estimation of the extent of inhibitory effects obtained (Table I).

Results. The findings clearly indicate that growth of this tumor is markedly inhibited by Vincaleukoblastine, Leurosine, and by the crude plant extract. However, the remaining alkaloids, Virosine, Lochnerine, Perivine, and Catharanthine and Vindoline, all proved inert in the dosages applied.

The growth-inhibitory effects of Vincaleukoblastine and of Leurosine are not accompanied by significant toxicity as evidenced by effects on host body-weight or upon host survival. It would, therefore, appear that these agents may be expected to have an acceptable margin of safety for clinical trial.

Summary. Crude extract of *Vinca rosea* as well as the alkaloids, Vincaleukoblastine and Leurosine markedly inhibit growth of a heterologously transplanted human choriocarcinoma in the hamster cheek-pouch. The host tolerance to these agents suggests their potential clinical usefulness.

The able technical assistance of Messrs. Howard Erwin, Charles K. Turner, and James Brice is gratefully acknowledged.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Bioflavonoids and Survival Time of Mice Exposed to Multiple Sublethal Doses of X-Irradiation.* (26084)

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Disagreement exists regarding the effects of flavonoids on the mortality of animals following exposure to a single lethal dose of total body x-irradiation. Rekers and Field(1, 2) reported an improved survival rate for irradiated dogs administered rutin or other flavonoids. Clark *et al.*(3) obtained increased survival of guinea pigs treated with calcium flavonate. Sokoloff and co-workers (4,5) found that CVP, a complex mixture of flavonoids separated from citrus waste, significantly reduced the mortality of rats administered a single lethal dose of total body x-irradiation. Opposed to these findings are the negative results of other workers who reported that rutin(6-11), CVP(10) and other flavonoids(10,11) had no significant effect on radiation mortality following exposure to a single lethal dose of total body x-irradiation in mice, guinea pigs and rats. Since previous findings indicate that certain dietary factors which have little if any protective effect in animals exposed to a single lethal dose of total body x-irradiation prolong survival in animals administered multiple sublethal doses of total body x-irradiation(12,13); experiments were undertaken to determine the effects of flavonoids on length of survival following exposure to multiple sublethal doses of total body x-irradiation in the mouse.

Procedure. The basal ration employed in the present experiment consisted of cerelose, 59%; casein,† 24%; cottonseed oil, 10%; salt mixture,‡ 5%; cellulose,§ 2%; and the following vitamins per kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; Vit. B_{12}, 150 μg; 2-methyl-1,4-naphthoquinone, 5 mg; choline chloride, 2 g; Vit. A, 5000 U.S.P. units; Vit. D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of cerelose.

Male mice of the Webster strain were selected at 11 to 14 g in body weight and were divided into comparable groups consisting of 40 animals per group. These were placed in metal cages with raised screen bottoms (5 animals per cage) and were fed either the basal ration or the basal ration plus the supplements listed in Table I. The supplements were added to the basal ration in place of an equal amount of cerelose. Food and water were provided *ad libitum.* The animals were fed daily and all food not consumed 24 hours after feeding was discarded. After 6 weeks of feeding 10 of the mice in each dietary group were selected at random to serve as non-irradiated controls. The remaining mice received an exposure of 200 r total body x-irradiation which was repeated once weekly until a total dose of 1200 r (6 exposures) had been administered. Animals were continued on their respective diets for 135 days after the first x-ray exposure or until death, whichever occurred sooner. All mice that died before the 4th x-ray exposure were not included in the tabulation of data. Data were obtained as to the day after first x-ray exposure on which 50% of the animals in each group were dead and average length of survival on each of the diets employed.

The following radiation factors were employed: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus

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* This investigation was supported in part by research grants from Nat. Cancer Inst., P.H.S., N.I.H., and contract with the Office of the Surgeon General, Dept. of the Army.

† Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Solka Floc, Brown and Co., Boston, Mass.

TABLE I. Effects of Flavonoids on Survival Time of Mice Exposed to Multiple Sublethal Doses of Total Body X-irradiation.*†

Dietary group	No. of animals	Day after 1st x-irrad. when 50% of animals in group were dead	No. surviving‡	Avg length of survival after 1st x-irrad., days§‖
Basal ration	26	39	5	66.6 ± 8.2
Idem + following supplements:				
2% hesperidin	28	40	7	63.0 ± 9.0
4% "	30	45	2	51.1 ± 4.4
2% lemon bioflavonoid complex	30	54	4	63.7 ± 5.6
4% *idem*	30	51	3	61.6 ± 5.5
2% naringen	30	66	8	81.4 ± 6.8
4% "	29	102	10	91.2 ± 8.0
2% hesperidin methyl chalcone	27	52	7	72.3 ± 7.8
4% *idem*	27	>135	15	107.3 ± 7.5
2% rutin	28	83	9	87.6 ± 7.4
4% "	28	118	11	101.9 ± 7.1

* Each group initially consisted of 30 mice. Animals that died before the 4th x-irradiation were not included in the Table.

† Hesperidin (Hesperidin Purified, Product No. 165), lemon bioflavonoid complex, naringen, and hesperidin methyl chalcone employed in the present experiment were kindly provided by Mr. W. E. Baier and Dr. G. H. Joseph of Sunkist Growers, Ontario, Calif. Rutin (Rutin N.F.X.) was obtained from the Meer Corp., New York.

‡ The experiment was terminated 135 days after 1st x-irradiation.

§ Data were calculated on the basis of an avg survival time of 135 days for animals alive at termination of experiment.

‖ Including stand. error of mean calculated as follows: $\sqrt{\dfrac{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}}{n-1}} \Big/ \sqrt{n}$, where "x" equals length of survival after 1st x-irradiation and "n" is No. of observations.

a Cu parabolic filter‖; HVL, 2.15 mm Cu; target distance to top of box, 82 cm; and dose rate, 15.6 r per minute (measured in air, at top of box). The animals to be irradiated were placed in a wooden box divided into 60 equal compartments 1¼″ wide, 3″ long, and 1½″ deep. The partitions and top were made of ⅛″ cellulose acetate sheeting; and top and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically driven turntable to ensure equivalent irradiation.

Results. Significant differences were observed between the various dietary groups as to the day on which 50% mortality occurred and average length of survival following exposure to multiple sublethal doses of total body x-irradiation (Table I). Whereas hesperidin and lemon bioflavonoid complex at levels of 2% or 4% of the diet had little if any effect on length of survival, the day 50% mortality was attained or number of animals surviving, naringen, hesperidin methyl chalcone and rutin at levels of 2% or 4% of the diet resulted in increased survival over that of animals fed the basal ration alone. Findings were particularly significant in the case of animals fed the hesperidin methyl chalcone and rutin supplements at a 4% level in the diet. No significant differences were observed grossly among non-radiated mice in the various groups. Average body weight of radiated and non-radiated mice in the various dietary groups at different stages of the experiment is summarized in Table II.

Discussion. Present findings indicate that dietary supplements of naringen, hesperidin methyl chalcone and rutin significantly in-

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‖ A nonuniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand(20). The center of the filter had a thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

TABLE II. Effects of Flavonoids on Body Weight of Radiated and Non-radiated Mice.*

Dietary group	Avg body wt at time of 1st x-irrad. (g)†	Avg body wt of survivors‡ (g) Radiated	Non-radiated
Basal ration	27.4 (39)	27.7 (5)	41.1 (9)
Idem + following supplements:			
2% hesperidin	30.0 (39)	32.3 (7)	42.5 (10)
4% "	30.4 (40)	29.8 (2)	38.3 (9)
2% lemon bioflavonoid complex	29.0 (39)	28.1 (4)	46.3 (9)
4% *idem*	29.1 (40)	31.0 (3)	41.6 (10)
2% naringen	29.7 (40)	31.2 (8)	41.5 (10)
4% "	26.9 (39)	27.8 (10)	37.7 (10)
2% hesperidin methyl chalcone	28.3 (37)	29.3 (7)	36.6 (9)
4% *idem*	30.2 (36)	32.0 (15)	39.9 (9)
2% rutin	27.7 (38)	31.1 (9)	46.1 (9)
4% "	28.7 (37)	34.7 (11)	41.9 (9)

* Avg initial body wt of mice in the various groups ranged between 12.2 and 12.4 g. Originally 40 mice were employed in each group. After 6 wk of feeding, 10 mice in each group were selected at random to serve as non-irradiated controls; remainder were exposed to x-irradiation. Values in parentheses indicate No. of animals which survived and on which averages are based.

† First x-irradiation was administered after 42 days of feeding.

‡ Experiment was terminated 135 days after 1st x-irradiation. In the case of the non-irradiated mice, this was 177 days (135 + 42 days) after the start of feeding.

creased average survival time of mice exposed to multiple sublethal doses of total body x-irradiation but that other flavonoids were without activity in this regard. No data are available as to the factors responsible for these diverse results. It is of interest that whereas hesperidin methyl chalcone increased average survival time of mice exposed to multiple sublethal doses of total body x-irradiation, hesperidin *per se* was without activity in this regard. In view of the greater solubility of the hesperidin methyl chalcone(14), it is not unlikely that differences in solubility of the various flavonoids may have contributed to the diverse results. No data are available as to the *modus operandi* whereby the active flavonoids exerted their protective effect. It has been suggested that the deleterious effects of ionizing radiation in animals are due, at least in part, to excessive formation of peroxides(15,16). If such were the case, preventing or minimizing peroxide formation following radiation or decreasing the sensitivity of various tissues or enzyme systems to the peroxides thus formed might exert a protective effect. In view of the chelating and antioxidant properties of various flavonoids(17,18), it is not unlikely that one or more of these substances might have significant activity in preventing or minimizing peroxide formation following radiation exposure, thereby resulting in a radioprotective effect. This suggestion is in accord with the radioprotective effects recently obtained with the synthetic antioxidants, propyl gallate, DBH (2,5-di-tert-butyl-hydroquinone) and BHT (butylated hydroxy toluene)(19). It is possible that fat-soluble derivatives of flavonoids might be prepared that would be even more effective against radiation injury. Further studies to test these hypotheses are indicated.

Summary. Experiments were conducted to determine the effects of flavonoids on survival time of mice exposed to multiple sublethal doses of total body x-irradiation. Hesperidin and lemon bioflavonoid complex at levels of 2% or 4% in the diet had little if any protective effect. Naringen, hesperidin methyl chalcone and rutin at levels of 2% or 4% of the diet increased survival over that on the basal unsupplemented ration.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Estrogenic Effects of 18-Norestradiol -17β and 18-Norestrone. (26085)

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Several workers have studied the effects of structural modification on biological actions and effectiveness of phenolic estrogens. Huggins and Jensen have discussed significance of carbon 17 hydroxyl groups of estradiol and of various modifications, related particularly to oxygenation at carbon 6 or carbon 16 (1,2); they showed that 17-desoxyestradiol was an active estrogen, albeit a material of low potency, and that 6 and 16 oxygenation resulted in estrogens that had shallow dose-response curves, *i.e.*, increment of uterine growth per unit increase in dose was less for these *impeded* estrogens than for estrone or estradiol. In mouse studies it was shown that there may be a whole series of possible dose-response curve slopes, extending from steep estrone slope, through intermediate slope of estradiol-17β, to shallow slope of estriol(3); similar results were obtained from spayed rats(4,5) and Drill, Cook and Edgren(6) have discussed several miscellaneous series of steroids; their finding most germane to present discussion was that 16-halogenation of 3-methoxyestrone does not produce an *impeded* or shallow slope. Most of these structural modifications have involved addition, removal or transposition of oxygenated functions. Recently synthesis of 18-norestrone and 18-norestradiol-17β has been completed(7). The uterine growth stimulating effects of these materials will be discussed here.

Materials and methods. Technics employed here have been described previously. Uterine growth was determined from tests employing immature female mice that received test compound daily for 3 days and were sacrificed on fourth. At autopsy uteri were removed, cleaned of adherent tissues and weighed wet on a torsion balance(3).

Results and discussion. As discussed previously(3,4), estrone had steepest slope of compounds treated; remaining materials had slopes that were essentially similar (Fig. 1). Slopes for estradiol-17β and estrone were appreciably more shallow than those cited earlier(3); this probably is result of change in mouse strain between the two studies (unknown strain from small breeder to Carworth mice from Small Animal Farms, Des Plaines, Ill.). The extremely high potency for estradiol-17β is the result of slope of dose-response curve and low response level at which 2 materials were compared; at 50 mg uterine weight level estrone and estradiol-17β would be equal in potency. Removal of angular methyl group at carbon 13 results in a sharp decrease in potency for each compound (Table I) with the estradiol derivative about

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TABLE I. Dose Response Curves and Potency for Estrone, Estradiol-17β, 18-Norestrone and 18-Norestradiol-17β.

Compound	Curve	Potency*
Estrone	Uterine wt $= -.2 + 30.5$ (log 100 dose)	100%
18-norestrone	" " $= 5.9 + 11.6$ (log dose)	.028
Estradiol-17β	" " $= 17.4 + 12.6$ (log 1000 dose)	2500
18-norestradiol-17β	" " $= 8.3 + 13.0$ (log dose)	.058

* At response equivalent to about 100% increase in uterine wt over oil-treated controls.

twice as potent as the estrone. The more remarkable finding, however, is decrease in slope of dose-response curve for 18-norestrone, which was similar to that for estradiol-17β. Why such structural modifications as removal of angular methyl group at carbon 13 or incorporation of oxygenated functions at carbon 6 or carbon 16 should produce decreases in slope of dose-response curve is unclear at present time.

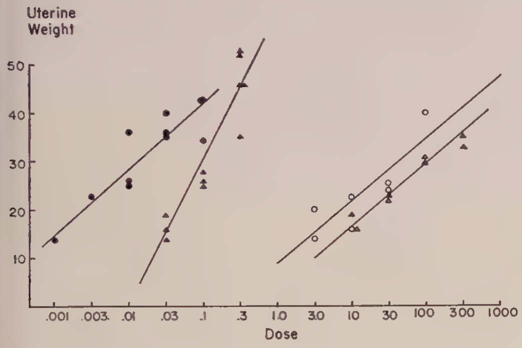

FIG. 1. Effects of estrone (▲), estradiol-17β (●), 18-norestrone (△) and 18-norestradiol-17β (○) on uterine growth in immature intact female mice. Each point represents one mean of 8-10 mice. Curves were fitted by the method of least squares.

Summary. Removal of the angular methyl group of estradiol-17β to form 18-norestradiol-17β results in a decrease in potency. A similar structural modification of the estrone molecule, forming 18-norestrone, produces both potency decreases and alterations in slope of the dose-response curve.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Absence of Sialic Acids and Other Carbohydrates in Crystalline Papain.*

(26086)

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Studies from this laboratory have been concerned for some years with the enzymic activity and structure of crystalline papain (1). Studies of the composition of this protein by Smith, Stockell and Kimmel(2) indicated that non-amino acid constituents were probably absent since the recovery of amino acids accounted satisfactorily for the weight and nitrogen content of the protein. Moreover, both Close, Moore and Bigwood(3) and Smith *et al.*(2) could detect no carbohydrate by the carbazole method. Recently, Loo, Lee and Flemming(4) reported that crystalline papain contains sialic acid by the direct Ehrlich method. It would be surprising indeed if sialic acid were present in a protein which

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* This investigation was aided by research grants from Nat. Inst. of Health, U.S.P.H.S.

gives a negative carbazole test, since sialic acid is generally associated with other carbohydrate constituents. Nevertheless, it was essential to reexamine papain for possible presence of various carbohydrate constituents. A study of the complete amino acid sequence of papain is currently in progress in this laboratory(1) and the presence of carbohydrate constituents could be of major importance.

Materials. Recrystallized papain was prepared from papaya latex by the method of Kimmel and Smith(5) and dialyzed against water. Specific activity (C_1) of this preparation was 2.0, among the highest found for any preparation(1). The purity of the enzyme has been established by a variety of methods(1).

Methods. Papain was heated in 0.05 N sulfuric acid at 90°C for 1 hour by the method used by Svennerholm(6) for determination of sialic acids. For chromatography, the hydrolysate was neutralized with barium hydroxide, and the barium sulfate precipitate and the soluble protein were separated by dialysis against several changes of water. The combined dialysates were collected and concentrated under vacuum below 28°C to a convenient volume for chromatography.

Aliquots were spotted on Whatman No. 1 paper and the chromatograms developed in the N-butanol-pyridine-water (6:4:3) solvent of Jeanes *et al.*(7). The chromatograms were sprayed with the thiobarbituric acid reagent of Warren(8) or the orcinol-trichloroacetic acid reagent of Klevstrand and Nordal (9), which was used for detection of sialic acids by Blix *et al.*(10).

Papain was also tested for sialic acids by both the direct Ehrlich method and by Bial's orcinol method as described by Werner and Odin(11).

Colorimetric analysis for hexoses was performed by the orcinol-sulfuric acid method, essentially as described by Winzler(12) for determination of protein-bound hexoses in serum proteins.

Results and discussion. A color yield corresponding to a sialic acid content of 1.2% was obtained by the direct Ehrlich method in agreement with the results of Loo *et al.*(4) who reported 1.1% sialic acid by the same method. However, sialic acids could not be detected chromatographically in aliquots of papain hydrolysates equivalent to 0.7 to 0.8 μmoles of papain, either by the thiobarbituric acid reagent or the orcinol-trichloroacetic acid reagent. In addition, the sialic acid content of papain was found to be less than 0.05% with Bial's orcinol method.

Examination of papain by the orcinol-sulfuric acid method showed that this protein contains less than 0.05% hexose when compared with a standard solution containing equimolar quantities of galactose and mannose.

Both the direct Ehrlich method and Bial's orcinol method for the determination of sialic acids lack specificity. Bial's orcinol reagent develops significant color with pentoses and, to a lesser extent, with hexoses. The orcinol-sulfuric acid reagent produces color reactions with hexoses, pentoses and uronic acids. The negative results with these last two tests indicate the absence of all these sugars as well as of sialic acids from papain.

It is evident from our previous and present studies that papain appears to be a simple protein devoid of carbohydrate constituents.

Summary. Studies of crystalline papain indicate that this protein does not contain carbohydrate. A report that sialic acid is present in this protein could not be confirmed by methods which are specific for these substances.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Fluorescent Antibody Technic for Sero-diagnosis of Schistosomiasis in Humans.* (26087)

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The diagnostic value of fluorescent antibody procedures has been adequately demonstrated in a wide variety of infectious diseases (1,2,3,4,5,6). Except for a study by Jackson (7,8), conducted to locate antibody binding sites in or about *Trichinella spiralis* and *Nippostrongylus muris* in infected animals, the fluorescent antibody technics have not been applied heretofore to the field of helminthology.

This report describes a fluorescent antibody test developed for laboratory diagnosis of schistosomiasis. The procedure was evaluated with human sera and results compared with those obtained by the slide flocculation test described recently by one of us(9).

Materials and methods. During preliminary investigations, eggs, miracidia, cercariae and adults of *Schistosoma mansoni* were stained by fluorescein tagged antisera. Brightest fluorescence and greatest uniformity of results were obtained with cercariae. Therefore, *S. mansoni* cercariae, freshly emerged from laboratory infected *Australorbis glabratus* snails, were used throughout these studies.

Human sera from 227 well-documented cases were tested by the fluorescent antibody test. Of these, 206 were also tested by the slide flocculation test. All schistosomiasis sera were obtained from persons in whom the diagnosis had been established by demonstrating the eggs in stools or urine. Some of the sera had been treated with merthiolate and others showed obvious signs of bacterial contamination. However, neither merthiolate in the amounts used for serum preservation nor the contamination seemed to interfere greatly with the reactions with either test. To determine the specificity of the reaction, sera from individuals with proven trematode, cestode, nematode, protozoan, mycotic and bacterial infections were tested. To test whether the presence of auto-antibodies would give rise to false positives, sera of patients with lupus erytemathosus were included. To test whether the positive reactions obtained in schistosomiasis patients might be due to nonspecific factors deriving from liver damage, a few sera from persons with other diseases of the liver were also included. Normal sera were obtained from healthy donors undergoing physical examination as candidates for appointment to the U. S. Military Academy. All sera were heated at 56°C for 30 minutes before being tested.

The indirect technic using a labelled goat antihuman globulin was employed. Dilution of sera and globulins were prepared in 0.01 M phosphate buffered saline at a pH of 7.2 (PBS). Labelled antihuman globulin was prepared by conjugation with fluorescein-iso-

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* The authors wish to express their appreciation to Major L. M. Muschel and Miss C. C. Campbell, Walter Reed Army Inst. of Research, Dr. W. De Witt, Lab. of Tropical Diseases, N.I.H., and Dr. I. G. Kagan, Communicable Disease Center, U.S.P.-H.S. for providing sera from patients with proven infections and from healthy individuals. Thanks are also due to Dr. E. H. LaBree and Mr. Earl Fife, Walter Reed Army Inst. of Research for useful suggestions in connection with use of the fluorescent antibody technic.

thiocyanate(10). Prior to use, each batch of labelled antibody was absorbed twice with powdered liver and was titrated with known immune and normal sera. The highest dilution of conjugate giving brilliant fluroescence of cercariae with *S. mansoni* antiserum and essentially no fluorescence with normal serum was used. In most instances a dilution of 1 to 2 satisfied these conditions. Fluorescent microscopy was accomplished by use of a Zeiss ultra-violet light assembly and microscope with dark field condenser and appropriate filters.

Test procedure. All reactions were carried out in 13 x 100 mm test tubes to prevent drying of the cercariae. For each test, 1 ml of cercarial suspension containing between 200 and 400 cercariae was added to 1 ml of 20% formalin. After 5 minutes the cercariae were washed twice in PBS by centrifugation at approximately 100 × G. One-tenth ml of test serum was added to the sedimented cercariae, and incubated for 30 minutes at room temperature (22°-24°C) while being gently agitated on a slide rotator (110 rpm). The cercariae were then washed twice with PBS and treated with 0.1 ml of diluted fluorescein-labelled antihuman globulin for 15 minutes on the slide rotator. The cercariae were again washed twice in PBS and suspended in 0.1 ml of 90% buffered glycerine. A small drop was placed on a micro-slide, mounted with a glass coverslip and examined under the ultraviolet light. The following controls were used: 1) Cercariae treated with 10% formalin, 2) Cercariae treated with antisera only, 3) Cercariae treated with normal serum followed by treatment with fluorescein-labelled antiglobulin, 4) Cercariae treated with normal serum only, 5) Cercariae treated with known positive serum followed by treatment with fluorescein labelled antiglobulin, and 6) Cercariae treated with fluorescein labelled antiglobulin only. Only cercariae which were treated with sera from known schistosomiasis patients revealed fluorescence following application of antihuman globulins. Positive reactions varied from strong to weak fluorescence. Degree of fluorescence was estimated according to criteria described previously (6).

To determine whether the fluorescence of cercariae treated with sera of schistosomiasis patients was due to a true antigen-antibody reaction, absorption of the antibodies from 3 sera which had produced bright fluorescence and positive flocculation reactions were attempted by treating these sera with an excess of cercarial antigen adsorbed onto cholesterol-lecithin crystals. Precipitates which were produced were removed by centrifugation and the supernatant was retested by the fluorescent antibody reaction. When retested, these sera were negative for presence of fluorescent antibodies.

Preparation of the antigen emulsion for the slide flocculation test and the performance of the test have been described(9).

Results. The results obtained with the 2 serological tests are summarized in Table I. The findings with sera from schistosomiasis patients illustrate the relative sensitivity of the 2 procedures. The findings with sera from patients with other conditions and from healthy individuals provide an index of the relative specificity of the 2 tests. Extensive cross reactions were obtained in both tests with sera from trichinosis patients. No significant difference in sensitivity and specificity of the 2 tests was observed.

Discussion. It is evident from our results that use of intact *S. mansoni* cercariae as an antigen and fluorescein-labelled antiglobulin as an indicator of cercaria-antibody coupling is a wholly feasible procedure. Drying of cercariae renders the test unsatisfactory since nonspecific staining is associated with desiccation of the cercariae.

The fact that sera reactive to the fluorescent antibody technic became nonreactive after absorption with soluble cercarial antigen indicates that this is a true antigen-antibody reaction. When the same absorbed sera were retested by the slide flocculation test, one was negative and two gave a doubtful reaction.

The occurrence of cross-reactions with sera from patients with *S. japonicum* and *S. hematobium* infections is consistent with findings with other tests and emphasize the close antigenic relationship of the schistosomes. The cross-reaction with sera from individuals with

TABLE I. Comparison of Results Obtained with fluorescent Antibody and Slide Flocculation Tests in Human Sera.

	Fluorescent antibody tests				Slide flocculation tests			
		Reaction				Reaction		
Diagnostic status	No. tested	Positive	Doubtful	Negative	No. tested	Positive	Doubtful	Negative
Schistosomiasis mansoni	88	67	13	8	72	59	9	4
Schistosomiasis japonicum	1	1	0	0	1	1	0	0
Schistosomiasis haematobium	18	12	5	1	18	11	4	3
Total schistosomiasis	107	80(75)	18(17)	9(8)	91	71(78)	13(14)	7 (8)
Clonorchiasis	1	0	0	1	1	0	0	1
Opisthorchiasis	1	0	1	0	1	0	0	1
Fascioliasis	2	0	1	1	2	0	2	0
Paragonimiasis	1	0	0	1	1	0	0	1
Echinococcosis	15	2	2	11	15	1	1	13
Trichinosis	11	9	0	2	11	8	2	1
Leishmaniasis	9	0	0	9	9	0	0	9
Histoplasmosis	3	0	0	3	3	0	0	3
Coccidioidomycosis	3	0	0	3	3	0	0	3
Blastomycosis	2	0	0	2	2	0	0	2
Syphilis	12	0	2	10	12	0	4	8
Tuberculosis	11	0	0	11	11	0	3	8
Liver cirrhosis	2	0	0	2	2	0	0	2
Hepatitis	3	0	0	3	3	0	0	3
Lupus erytematosus	5	0	0	5	5	1	2	2
Healthy controls	39	1(3)	3(8)	35 (90)	34	0(—)	2 (6)	32(94)

The figures in parentheses indicate %.

trichinosis has been observed before(9,11) and does not seriously handicap the test as a diagnostic tool because geographical distribution of the two diseases is fairly distinct.

The results suggest that the fluorescent antibody test may provide a relatively simple and reliable procedure for the diagnosis of schistosomiasis of particular value to small laboratories, since the procedure is not expensive, materials can be made available from commercial sources, the actual reading of the tests is very rapid and results can be obtained within a few hours after collection of blood. Increasing experience with the observation of cercariae by the use of fluorescent microscopy should reduce the number of doubtful reactions. However, the reliability of the test must await more complete documentation. Therefore, it is too early to speculate on the immunological significance of the antibodies detected by these means and whether the same antibodies are responsible for the flocculation and the fluorescent antibody reactions.

Summary. Use of fluorescein-labelled antiglobulin as an indicator of cercarial antibody is described. Sensitivity of the procedure appears to be great for all human schistosome infections. While non-specific staining occurs regularly with sera from trichinosis patients, the specificity with other sera suggests that this procedure may be useful in serological diagnosis of human schistosomiasis.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Infection of *Australorbis glabratus* with *Schistosoma mansoni* under Bacteriologically Sterile Conditions.* (26088)

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Australorbis glabratus, major snail vector of human schistosomiasis in the Western Hemisphere, can be secured free from demonstrable bacteriological contaminants(1) and grown axenically for protracted periods *in vitro*(2). Although axenic snails appear normal grossly and histologically, it seemed important to determine their susceptibility to infection with *Schistosoma mansoni* and their capacity to sustain complete larval development of this parasite.

Materials and methods. 1. *Strains of A. glabratus.* Four Puerto Rican (PR) strains provided eggs: PR-2, maintained in our laboratory since 1957(2); PR-2A and PR-2B, derived from the same Puerto Rican colony as PR-2 but kept in this laboratory since Aug. 1959; PR-3, established in Aug., 1959, from wild caught snails from Padin's Pond, San Juan. Stock colonies of PR-2 and PR-2A snails were maintained as described(2); colonies of PR-2B and PR-3 were maintained in a similar manner except that aquaria contained commercial potting soil (150 g/10 liters) in addition to marble chips, and tap water passed through a charcoal column instead of distilled water. 2. *Technics for axenic cultivation.* Mature snail eggs were dissected from egg-masses, disinfected, placed in individual screw-cap tubes and permitted 3-4 days to hatch in a balanced salt solution containing penicillin G., streptomycin sulfate, $NaHCO_3$, and phenol red(1). This fluid was then replaced with 5 ml/tube of filtered, autoclaved (15 lb/30 min) aquarium water to which all of the foregoing were added except streptomycin(2,3). This is referred to as maintenance fluid. All snails were fed a diet of autoclaved brewer's yeast and formalin-killed *Escherichia coli*(2). Snails which reached or exceeded 2 mm in diameter after the first feeding were refed after transfer (usually at 14-25 days) to individual 160 ml screwcap bottles containing 25 ml of fresh maintenance fluid. At weekly intervals thereafter fluid was replaced, and snails refed. When snails reached 5-6 mm in diameter, allotments of maintenance fluid and food per snail per week were doubled for the balance of experiments. All snails were kept in incubators at 27-28°C and were measured weekly by ocular micrometer. Sterility tests were done prior to each feeding by inoculating aliquots of fluid and fecal debris from each snail into tubes of liquid thioglycollate medium which were incubated at 28-32°C for one week and at room temperature for another week. These tests were supplemented periodically by parallel use of nutrient broth and blood agar media. 3. *Securing bacteriologically sterile miracidia of S. mansoni.* Webster strain mice, each infected intraperitoneally with 100 cercariae of a Puerto Rican strain of *S. mansoni*, were supplied by Dr. Henry van der Schalie, Univ. of Michigan. Livers were removed aseptically from 3-6 mice infected 8-10 weeks previously, pooled, and processed as described by Lee and Lewert(4) with the following modifications: a) instruments, glassware, and solutions were sterile; b) miracidia were hatched under light in volumetric flasks containing sterile aquarium water and 100 units/ml penicillin. Miracidia were transferred *en masse*, within 30 minutes of hatch, to a small Petri dish and picked out by Pasteur pipette under low magnification. Aliquots of miracidial suspension in each experiment were inoculated into tubes of liquid thioglycollate medium and of nutrient broth; incubation for 2 weeks disclosed no contaminants. 4. *Exposure of axenic A. glabratus to miracidia.* Most snails were exposed to infection at 2-3 weeks of age (mean diameter 2.1-2.4 mm),

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* This investigation was supported in part by grant from Nat. Inst. of Allergy and Infect. Diseases, and by a Senior Research Fellowship from U.S.P.H.S.

just before transfer from tubes to bottles. Prior to exposure, maintenance fluid was removed from tubes and replaced by 3 ml of sterile aquarium water containing 100 units/ml penicillin (pH = 7-8). Counted miracidia were introduced in 1-2 drops into each tube by Pasteur pipette, caps were tightened, and tubes placed in an incubator at 27-28°C. The inner glass door of the incubator was closed, but the outer door was left open during exposure period to allow a constant low level of illumination from artificial lights of the room. Snails were removed from tubes after 18-20 hours, transferred to individual bottles, and maintained as described earlier. For exposure to infection of older snails (60-66 days, mean diameter 6.7-6.9 mm), maintenance fluid was discarded, 10 ml per bottle of sterile aquarium water containing 100 units/ml penicillin was substituted, and counted miracidia were added. Other exposure conditions were as described above. After exposure, maintenance fluid and food were substituted and all snails were maintained in dark incubators (27-28°C). Bottles were removed from incubators only for feeding, measuring, or examining snails. As indirect controls in each experiment, aquarium-reared snails (all strain PR-2), comparable in size to axenic snails, were individually exposed to infection in tubes or bottles under conditions identical to those outlined for axenic snails, including batch and number of miracidia. After exposure, control snails were kept in groups in aerated battery jars (26°C) supplied with watercress; ceiling-level fluorescent lights were kept on at all times since natural light was excluded from the room. 5. *Examination of snails for schistosome infection.* Snails *in vitro* were examined 8-10 days after exposure for presence of sporocysts using transmitted light, 45× magnification, and viewing each snail from its ventral aspect. Snails were examined for cercarial emergence every 1-2 days starting about 20 days post-exposure. Bottles were placed under a desk lamp for about 2 hours, then examined individually for cercariae using a 7× lens and right-angle, narrow beam, transillumination. Snails shedding cercariae were usually reexamined every 3-4 days thereafter to determine duration of cercarial production. In one experiment snails exposed to infection were fixed in Newcomer's solution in groups of 2-3 starting 24 hours after exposure, and at weekly intervals thereafter for 8 weeks, and stained 8 μ serial sections of each specimen were examined for parasite-development. 6. *Exposure of mice to cercariae from bacteriologically sterile A. glabratus.* Although cercariae were shed regularly and apparently survived well in the bicarbonate-containing maintenance fluid in stoppered bottles, newly-emerged cercariae became inactive or moribund within 2-3 hours after being decanted into open Petri dishes. This phenomenon appeared to be related to a rapid alkaline shift in pH. Accordingly, when infectiousness of cercariae for mice was to be tested, maintenance fluid was removed and replaced with 10 ml sterile aquarium water containing 100 units/ml penicillin, and the bottles then exposed to light. When a suitable density of cercariae had accumulated, the fluid from each bottle was decanted into a Petri dish from which counted cercariae were transferred by Pasteur pipette to 15 x 75 mm tubes. This procedure was done without sterility precautions since anaesthetized white mice (20 g) were immediately exposed to the cercariae for 1-2 hours by the tail immersion method(4). Mice were sacrificed and examined for adult worms and the presence of viable eggs about 8 weeks after exposure.

Experimental results. Data summarized in Table I show that infections in partly-grown *A. glabratus* were readily established under bacteriologically sterile conditions. Of 66 axenically-reared snails exposed to miracidia, sporocysts were visible in 53 individuals 8-10 days later, and 61/63 survivors (97%) shed cercariae by 33 days. Mortality during this period among infected, bacteriologically sterile, snails was low and comparable to that among uninfected axenic controls. On the other hand, mortality among schistosome-exposed aquarium-reared snails was much higher (67%) than among the bacteriologically sterile snails (<5%). Losses among the former generally occurred before infections reached maturity (Table I), and ranged

TABLE I. *Schistosoma mansoni* Infections in Bacteriologically Sterile and in Aquarium-Reared *Australorbis glabratus*.

Axenic snails exposed							Post-exposure			
				Per snail			Day 25-27		Day 30-33	
Exp. No.	Strain	Age (days)	Mean diam. & range (mm)	No. miracidia	Vol (ml)	No. snails	Snails alive	Cum. No. shedding	Snails alive	Cum. No. shedding
HP-2	PR-2A	13	2.3 (2 -2.5)	5-10	3	13	13	10	13	13
HP-3a	PR-2B	14	2.1 (1.8-2.5)	10	3	20	20	19	18	18
-3b	PR-2	25	2.4 (1.8-2.6)	10	3	6	6	1	6	6
-3c	PR-2A	66	6.9 (6.6-7.2)	15-20	10	5	5	2	4	3
-3d	PR-2A	60	6.7 (6.2-7.0)	15-20	10	9	9	6	9	8
HP-4	PR-3	17	2.2 (2 -2.6)	10	3	13	13	4	13	13
					Totals	66†	66	42	63‡	61
					%	—	100	64	95	97
	Aquarium-reared snails exposed*				Totals	48	25	9	16	13
					%	—	52	30	33	81

* All strain PR-2.
† 53/66 had 1-3 visible sporocysts per snail 8-10 days after exposure.
‡ Loss of 3 snails accounted for by 1 death and 2 contaminated.

from 37% to 93% in the several groups exposed.

Comparison of growth rates for infected snails and uninfected controls, maintained under identical bacteriologically sterile conditions, was made in experiments HP-2, HP-3a, and HP-4. These data disclosed that infected snails consistently grew at a more rapid rate than controls, and that these differences regularly became apparent within the first week after exposure. Representative data from experiment HP-3a are shown in Table II.

The course of development of *S. mansoni* in bacteriologically sterile *A. glabratus* appeared normal as judged by tissue sections (Fig. 1), and the cercariae produced *in vitro* were indistinguishable in appearance and behavior from those shed by aquarium-reared snails. Direct counts of cercariae done on the entire contents of a few bottles disclosed that as many as 500-1000 cercariae emerged from individual snails during 2 hour periods at peak of cercarial production.

Bacteriologically sterile cercariae proved to be infectious. Seven mice, each exposed to 100-150 cercariae derived from several snails in Exp. HP-2, were found to have mature schistosomes 8 weeks later. The presence of adult worms was confirmed by direct observation of portal and mesenteric blood vessels or by perfusion of the liver. Gross mottling and enlargement of the liver was evident in all mice, large numbers of eggs were seen in press preparations examined microscopically, and viability of these eggs was confirmed by hatching miracidia from ground livers. The entire cycle was then repeated by recovering these miracidia under sterile conditions and successfully infecting 7/8 axenically-reared *A. glabratus*.

TABLE II. Mean Shell Diameter (mm) of Schistosome-Infected and Uninfected *A. glabratus* Maintained under Sterile Conditions (Exp. HP-3a).

	Days post-exposure						
Snails	0	9	16	22	29	36	43
Infected*	2.1	4.2	5.1	5.5	5.8	6.0	6.2
Uninfected†	2.1	3.3	4.4	5.0	5.2	5.6	5.8

* N = 20 initially; 18 after day 29. † N = 14.

The first emergence of cercariae from the bacteriologically sterile snails in HP-2, 3, and 4 usually occurred 25-30 days after exposure, although in a few instances cercariae were first seen as early as the 22nd day or as late as the 33rd day. However, in one experiment in which newly-hatched, previously unfed, axenic *A. glabratus* were exposed to infection with *S. mansoni* somewhat different results were obtained. Fourteen such snails (1-4 days old, .65-.85 mm diam., strain PR-3) were each exposed to 10 miracidia in 3 ml under described conditions of exposure and subsequent sterile maintenance. Only 6/14 (43%) became infected; 3/6 first shed cercariae 24-31 days after exposure, but cercarial emergence from the remaining 3 did not occur until 42-49 days after exposure.

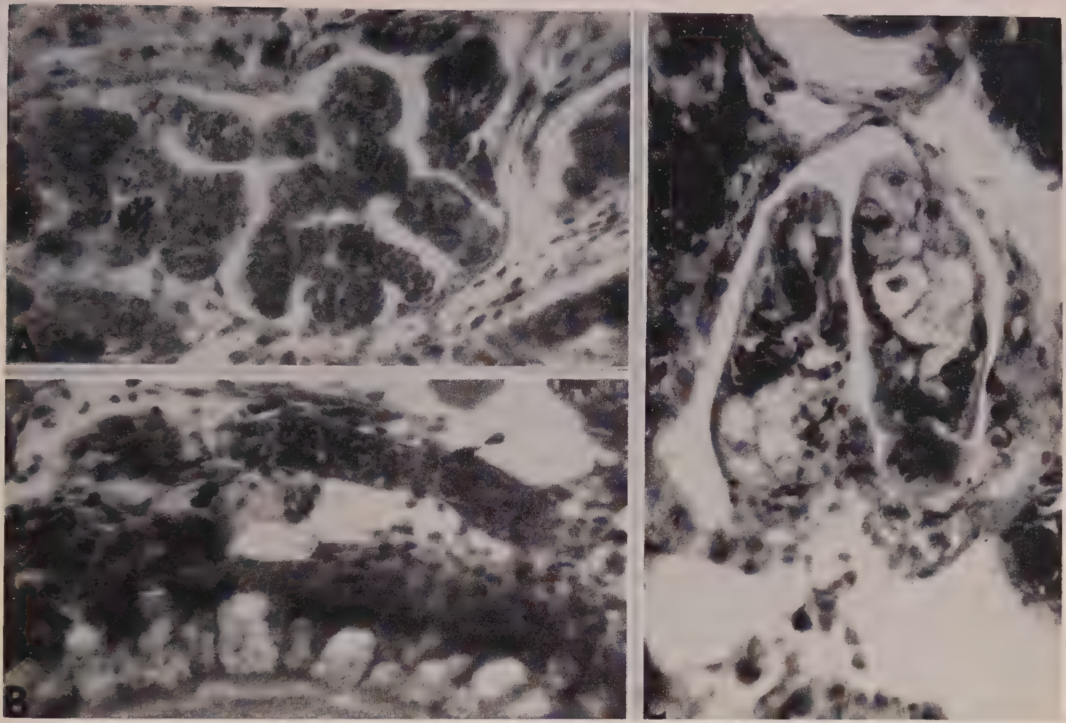

FIG. 1. Development of *S. mansoni* in bacteriologically sterile *A. glabratus* (Exp. HP-4). A. Mother sporocyst in foot 8 days post-infection. B. Daughter sporocyst in liver 15 days post-infection. C. Mature cercariae in liver 27 days post-infection. A and B stained with haematoxylin-eosin; C with haematoxylin-azure II-eosin. All sections ×425.

While a small proportion of bacteriologically sterile snails infected with *S. mansoni* shed cercariae for only 3-10 days, most produced cercariae for several weeks. About 15% produced cercariae for 50 days or more, and 2 individuals regularly liberated cercariae for 87 and 98 days. Some snails produced cercariae until they died, but in most instances several days or several weeks elapsed between last emergence and death of the snail host. When maintained under sterile conditions for extended periods following onset of cercarial shed, infected snails tended not to survive as long as uninfected controls.

Discussion. A. glabratus grown in the absence of demonstrable contaminants were readily infected with *S. mansoni* and served as suitable hosts for normal development of the extra-mammalian phase of the parasite life-cycle. The sequence of events from miracidial exposure to cercarial emergence was consistent with that reported in the literature for snails reared under conventional conditions. The unusually high mortality noted among infected aquarium-reared snails is unexplained but may have resulted from a) secondary microbial infections, b) differences in strain, nutritional status, or handling methods, or c) constant exposure to light. The latter possibility was explored experimentally, but no correlation was observed in mortality among infected snails kept in the light or in the dark.

The experimental demonstration that *S. mansoni* develops normally in snails kept in the dark may have epidemiological significance since the absence of light in covered irrigation conduits or similar situations would probably not inhibit parasite development in infected snails. It may also be noted in this context that, in the absence of information as to the significance of light in terms of successful miracidial penetration, low illumination was provided when snails were actually being exposed to miracidia. A preliminary experiment with aquarium-reared snails has since shown, however, that penetration of miracidia into *A. glabratus* can occur in total darkness, conjectures to the contrary(5) notwithstanding.

It is of interest that schistosome-infected snails grew somewhat more rapidly than uninfected ones under sterile conditions. Parasitization by certain larval trematodes is said to cause excessive growth of some snails (6, for review), and it has been suggested that such snails apparently overcompensate by voracious feeding for nutrients supposedly withdrawn by the parasite(7). However, under the bacteriologically sterile conditions of the present study, parasitized and control snails were fed the same amount of food per week and little or no food remained unconsumed in either case. If the parasites were "withdrawing" food, parasitized snails might have been expected to be smaller than control snails, while in fact the converse was observed. It seems doubtful that "parasitic castration" could have been significant in this since most of the snails were small and sexually immature, and since the differences in growth become apparent within a week of infection.

Successful cultivation *in vitro* of schistosome-infected *A. glabratus* opens new areas for investigation, particularly insofar as the bacteriologically sterile nature of the system provides a source of uncontaminated larval stages, including cercariae, for possible use as antigens or for studies on cultivation *in vitro*.

Summary. *Australorbis glabratus,* reared axenically to 2-7 mm before exposure, were found to be highly susceptible to infection with *Schistosoma mansoni* in the absence of demonstrable bacteriological contamination. Larval development was normal in all observable respects, and cercariae infectious for mice were produced after normal periods of incubation. Maintenance of these snails in the dark for extended periods did not inhibit larval development. Mortality was very low among schistosome-infected snails maintained in the sterile state. Infected snails tended to grow more rapidly than uninfected snails when both were fed identical rations under controlled, bacteriologically sterile conditions.

I am indebted to Dr. C. Pan for preparation and interpretation of tissue sections, to Dr. E. H. Michelson for securing some of the strains of snails, and to Ann R. Schork and Caryl A. Dunavan for technical assistance.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Effects of Amethopterin on Cell Growth and Viral Synthesis.* (26089)

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Mammalian cells have been found not to require any purine or pyrimidine for growth in Eagle's medium(1,2), but the presence of some form of folic acid is obligatory(3). A variety of cultivated mammalian cells have been found unable to proliferate in growth media containing amethopterin (4-amino-N^{10}-methylpteroyl-glutamic acid) an anti-

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* This work was supported by a grant from the National Foundation, New York City.

folic antagonist(1). The growth inhibitory effects of amethopterin, however, can be completely reversed with a mixture of adenine, glycine and thymidine(4,5). In the present investigation cell growth and viral synthesis was examined in cultures of HeLa cells made thymine-deficient by treatment with amethopterin supplemented only with adenine and glycine.

Materials and methods. The line of HeLa cells was originally obtained from Difco Laboratories, and routinely grown in Eagle's basal medium (EBM)(6) containing 10% human serum. Monolayer cultures for experiments were established in EBM and 10% equine serum (EBM10ES). On day 2 (2 days after initiation of cultures) cultures were washed with Hank's balance salt solutions, and the experimental medium introduced. This medium was renewed on day 4. On day 6 measurements of cell growth were made or the cultures were infected with one of the various viruses studied. The basic experimental medium consisted of EBM10ES supplemented with adenine (1 x 10^{-6}M) and glycine (3 x 10^{-4}M). To this medium, amethopterin (1 x 10^{-6}M) and/or thymidine (3 x 10^{-3}M) was added. Viruses included in this study were poliovirus type 1 (Mahoney), herpes simplex virus (Armstrong), and vaccinia virus (Mich. Dept. of Health). Poliovirus was titrated in tube cultures of HeLa cells and the 50% endpoint calculated by the method of Reed and Muench(7). Both vaccinia and herpes simplex viruses were assayed on chorioallantoic membranes of embryonated eggs according to the method of Beveridge and Burnet(8), and amount of virus present expressed as pock-forming units (PFU) per ml. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) fractions of the cell cultures were obtained according to the method of Schmidt and Thannhauser (9), and their phosphorus content determined according to the method of Fiske and Subbarow(10). Cellular protein was measured by the method described by Oyama and Eagle(11).

Results. Growth of Thymine-deficient cells. The effect of varying concentrations of amethopterin (AM) in presence of added adenine and glycine on growth of HeLa cells is shown in Table I. Cell growth was determined on the fourth day after introduction of the experimental media, and is expressed in terms of protein content of the cultures.

TABLE I. Growth of HeLa Cells as a Function of Amethopterin (AM) Concentration.

Concentrations of AM (M)	Experimental media	Day	Protein (μg)
0	—	2	340.1
0	—	6	635.6
10^{-8}	A, G	6	625.7
10^{-8}	A, G, T	6	626.6
10^{-6}	A, G	6	373.7
10^{-6}	A, G, T	6	611.7
10^{-4}	A, G	6	387.3
10^{-4}	A, G, T	6	609.9

Cell cultures cultivated in EBM10ES at 37°C for 2 days, then changed to experimental media and reincubated for another 4 days, when protein determinations were made. Experimental media: EBM10ES with A = adenine (1 × 10^{-5}M), G = glycine (3 × 10^{-4}M), and/or T = thymidine (3 × 10^{-3}M).

AM concentrations of 10^{-6} and 10^{-4} but not at 10^{-8}M, inhibited cell growth (Table I). Degree of inhibition at the 2 higher concentrations of AM was essentially the same. As shown in the same table, this inhibition was removed by addition of thymidine. In other experiments, addition of thymidylic acid instead of thymidine reversed the effect of AM. These results are in agreement with those found by Hakala and Taylor(5) for amethopterin, and by DeMars and Hooper(12) for aminopterin-treated HeLa cells.

In Table II are shown results of an experiment where determinations of cell number, RNA-P and DNA-P, as well as protein, were made.

The AM inhibition of cell growth is again apparent on day 6 if number of cells present in thymine-deficient cultures is compared with that of untreated or thymidine supplemented cultures. There is, in fact, a loss of cells in the thymine-deficient cultures at 6 days as compared to the untreated ones at day 2. This inhibition of cellular multiplication and loss of cells also has been observed by DeMars and Hooper(12) in aminopterin-treated cultures.

TABLE II. "Unbalanced" Growth of HeLa Cells in Presence of Amethopterin.

Experimental media	Day	Cell count $\times 10^6$	mg DNA-P $\times 10^{-10}$/cell	mg RNA-P $\times 10^{-10}$/cell	mg protein $\times 10^{-8}$/cell
—	2	3.32	16.0	48.1	44.1
—	6	6.23	16.8	44.2	41.5
AM, A, G	6	2.23	17.5	95.6	73.0
AM, A, G, T	6	5.95	17.1	45.3	43.4

HeLa cells were cultivated in EBM10ES.

After 2 days, the experimental media were added—EBM10ES with A = adenine (1×10^{-5} M), G = glycine (3×10^{-4} M), and/or T = thymidine (3×10^{-3} M), AM = amethopterin (1×10^{-6} M).

Cell count, protein and nucleic acid determinations were made on 4th day after introduction of experimental media.

The amount of DNA per cell is the same in all cultures, including those deficient in thymine (Table II). However, the deficient cultures have relatively more RNA and protein per cell.

These biochemical observations are supported by direct microscopic examination of the thymine-deficient cultures (Fig. 1). As compared to untreated or thymidine supplemented cultures, those starved of thymine show fewer cells and an absence of mitotic figures. These cells and their nuclei are markedly enlarged. The nuclear chromatin forms a coarse reticulum as compared to the more diffuse arrangement seen in the untreated cells. The nucleoli are enlarged and intensely basophilic.

Ability of thymine-deficient cells to support viral synthesis. Replicate cultures of AM-treated, AM-thymidine-treated, and non-treated HeLa cells were prepared according to the manner described above. These cultures were infected with a sufficiently large inoculum of vaccinia virus, herpes sim-

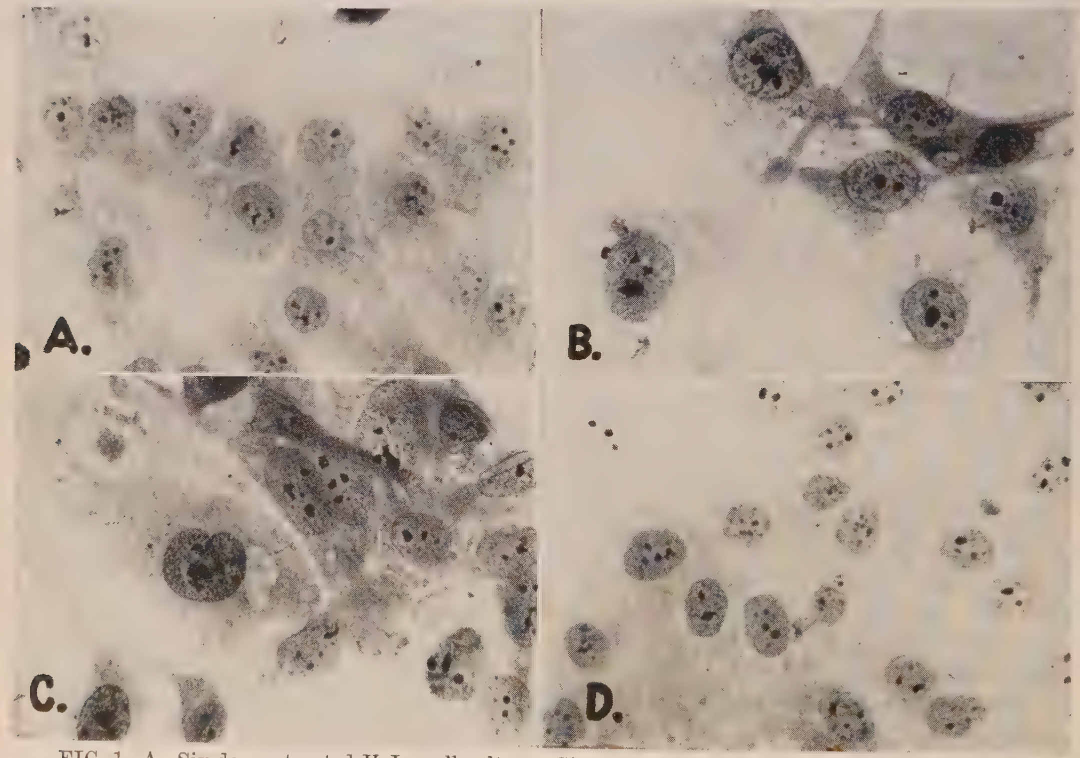

FIG. 1. A. Six day untreated HeLa cell culture. Giemsa. ×440. B. and C. Six day HeLa cell culture, treated from day 2 with AM. Giemsa. ×440. D. Six day HeLa cell culture, treated from day 2 with AM and thymidine. Giemsa. ×440.

TABLE III. Viral Synthesis in Thymine Deficient HeLa Cells.

Virus*	Control	Amethopterin treated	Amethopterin + thymidine treated
Vaccinia†	2.4×10^7§	3×10^6	1.8×10^7
Herpes simplex†	1.92×10^7§	1.7×10^6	2.61×10^7
Poliovirus Type 1‡	$10^{6.5}$‖	$10^{6.2}$	$10^{6.3}$

* Cell cultures incubated with vaccinia and herpes simplex viruses for 4 hr, and poliovirus for 1 hr at 37°C, then washed with Hank's balance salt solution to remove unadsorbed virus and reincubated after addition of experimental media. Total amount of virus was determined at 24 hr.

† Inoculated with approximately 60 PFU/cell.

‡ Inoculated with approximately 10 $TCID_{50}$/cell.

§ Pock-forming units/ml (PFU).

‖ Tissue culture infectious dose ($TCID_{50}$).

plex virus (Armstrong) or poliovirus type 1 (Mahoney) to insure infection of essentially all the cells. Total virus was determined 24 hours after initiation of infection.

The results shown in Table III indicate that in HeLa cells made thymine-deficient by AM, there was inhibition of viral synthesis of both vaccinia and herpes viruses, but not of poliovirus. As compared to the untreated infected control, inhibition of vaccinia and herpes simplex viruses amounted to 87.5% and 91.1% respectively. This inhibition was effectively removed by addition of thymidine to the AM-treated cultures. Although the synthesis of vaccinia virus was inhibited, these cultures showed cytopathic charges resembling those of virus-infected and non-treated cultures. Since the untreated herpes infected cultures showed little or no cytopathology at 24 hours, no definite effect on cytopathic response could be ascribed to the thymine-deficient state. Poliovirus induced cytopathology was the same in all 3 experimental cultures.

Discussion. In the present paper the effect of amethopterin, a folic acid antagonist, on growth of HeLa cells has been examined. Using conditions similar to those of Hakala and Taylor(5), it was found that the activity of this inhibitor can be restricted to a single metabolic pathway. That is, in a medium containing amethopterin supplemented with adenine and glycine, thymine synthesis and cellular multiplication are prevented. This inhibition can be reversed by thymidine or thymidylic acid. As observed here and by Hakala and Taylor(5), the thymine-deficient cultures synthesize less protein than normal. However, when the amount of protein, RNA and DNA are expressed on a per cell basis, it is seen that whereas protein and RNA continue to accumulate, DNA does not. This disproportionate increase in both protein and RNA relative to DNA is reminescent of the phenomenon of "unbalance growth" found in thymineless mutants of bacteria(13). DeMars and Hooper(12), relating total cellular protein to cell number, have recently reported similar results in HeLa cells made thymine-deficient with another folic acid antagonist aminopterin (4-aminopteroylglutamic acid).

The replication of several animal viruses in amethopterin-induced thymine deficient cells was studied and found to be dependent upon the type of nucleic acid contained in the virus. With DNA-containing viruses such as vaccinia and herpes simplex, viral synthesis is inhibited. The inhibition of viral synthesis is prevented by the presence of added thymidine or thymidylic acid. This suggests that the pathways of synthesis of viral or cellular DNA are quite similar, and once again emphasizes the qualitative similarities in the metabolism of virus-infected and uninfected mammalians cell(14). It further suggests, as postulated by Magee, Sheek, and Burrows, (15) that viral DNA is synthesized *de novo*. The inhibition of herpes simplex virus synthesis is of particular interest since the results further support the belief that it is a DNA virus. Newton and Stoker(16), have found in herpes-infected cells increased amounts of DNA which is believed to be non-viral. This increase in DNA content was observed not to occur in the inhibitor-treated herpes-infected cultures (to be reported).

Although the inhibitor prevented vaccinia virus synthesis, it did not interfere with the development of cytopathology. This may be attributable to the "toxic effect" described by Brown, Mayyasi, and Officer(17) for a strain of vaccinia virus. It is also possible that the action of this antagonist is similar to that of fluorophenylalanine in poliovirus-infected cells, where the amino acid analogue inhibited the synthesis of poliovirus, but not the occurrence of cytopathology(18).

With the RNA-containing poliovirus, no inhibition of virus synthesis or development of cytopathology was observed at concentrations of amethopterin that inhibited DNA synthesis. Thus it appears that active cellular DNA synthesis is not required in the synthesis of a RNA-containing virus.

Summary. Amethopterin, a folic acid antagonist, has been found to prevent multiplication of HeLa cells in Eagle's medium containing added adenine and glycine. This inhibition can be removed by addition of thymidine or thymidylic acid. The amethopterin-induced thymine deficiency specifically results in a cessation of cell division and a disparate increase in cellular RNA and protein to DNA content. Ability of amethopterin-induced thymine deficient cultures to support viral synthesis appears to be dependent upon the type of nucleic acid contained in the virus. With DNA-containing viruses such as vaccinia and herpes simplex, viral replication is inhibited, whereas the synthesis of a RNA-containing virus such as poliovirus is not.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Electrophoretic Studies of Serum Proteins and Glycoproteins During Early Stages of Experimental Tuberculosis.* (26090)

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Several studies have employed filter paper electrophoresis to determine changes in concentration of serum glycoproteins and proteins in clinical(1,2) and experimental(3) tuberculosis. Previous investigations have been primarily concerned with alterations in serum glycoprotein and protein patterns in well-established infections, and with the effects of therapy. The present study was undertaken to investigate the rate and extent of change in electrophoretic patterns in the early stages of an experimental infection.

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* Supported in part by a grant from Tuberculosis and Health Assn. of Los Angeles County.

TABLE I. General, Pathologic and Hematologic Data.

Group (days post-infection)	No. guinea pigs	Index of tuberculosis†					Tuberculin reaction§	Hemoglobin (%)*	Hematocrit (%)*
		Spleen	Liver	Lungs	Lymph nodes	Total*			
0 (nor)‖	25							12.9 ±.10	41 ±.3
8	19	.5	.0	.0	.0	.5 ±.11	1.2	12.7 ±.14	40 ±.4
15	13	.6	.1	.0	1.7	2.4 ±.30	1.1	13.0 ±.21	43 ±.6‡
19	16	1.0	1.0	.1	1.5	3.6 ±.24	1.6	12.0 ±.18‡	39 ±.5‡
29	8	2.4	.8	.8	3.1	7.1 ±.41	4.0	11.8 ±.13‡	39 ±.5‡
50	18	2.0	1.5	1.8	2.6	7.9 ±.42	3.8	11.5 ±.18‡	41 ±.5

* Including stand. error of mean.

† Each organ was assigned a value of 0 to 4 according to extent of macroscopic tuberculosis. Maximum possible extent of disease in each animal was 16. Index of tuberculosis is avg total for each group.

‡ P = <.01.

§ Avg tuberculin reaction by rating of 0-4+.

‖ nor = normal.

Experimental. Adult, virgin guinea pigs with an average weight of 750 g were found to be nonreactors to intradermal injection of 0.1 ml of a 5% solution of Old Tuberculin. They were inoculated subcutaneously in the inguinal region with 0.1 mg (moist weight) of *Mycobacterium tuberculosis,* strain B88. The animals were housed in an air-conditioned room in groups of 5 to 8 and were maintained on a diet of Purina rabbit pellets, supplemented with fresh greens and tap water. Groups of guinea pigs were sacrificed at intervals following inoculation. Two days prior to autopsy, the animals were again skin-tested. Blood samples were obtained by cardiac puncture under ether anesthesia. Skin tests were read and extent of macroscopic tuberculosis estimated by procedures previously reported(4). Chemical and hematologic determinations were made by methods described earlier(5).

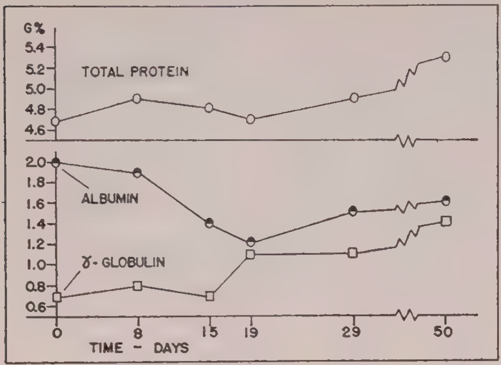
Electrophoretic analyses were carried out under conditions previously described(6) except that a current of 7.5 Ma was employed. Serum samples of 0.01 and 0.03 ml were used for protein and glycoprotein analyses, respectively. Strips were stained for protein with bromphenol blue, and for glycoprotein with the periodic acid-Schiff reagents. Absolute concentrations were determined by reference to total protein and total glycoprotein levels. Analysis of the data was made by standard statistical procedures, employing the *t* test (7).

Results. General, pathologic and hematologic data are presented in Table I. The inoculum employed caused an early conversion of dermal sensitivity and progressive disease. Significant decreases in hemoglobin concentrations were observed in the groups with the greatest amount of disease. Hematocrit levels exhibited some fluctuations. Alterations in electrophoretic patterns, however, cannot be attributed to changes in plasma volume.

Results of electrophoretic analyses are summarized in Fig. 1-4. The earliest significant change in protein patterns was the increase in α_1-globulin in the 8-day group. A further increase was noted 15 days post-inoculation. and the fraction remained significantly elevated throughout the experiment. Albumin levels were significantly decreased 15 days post-infection and remained so. Significant increases in γ-globulin concentrations were first observed in the 19-day group. Further elevation of the fraction was found in the 50-day group which resulted in a significant hyperproteinemia.

A significant increase in total glycoprotein was observed 8 days post-infection due to elevation of the protein-bound carbohydrates of the α-globulins. Maximal changes in protein-bound carbohydrates had occurred 15 days post-infection, α- and β-globulin fractions accounting for 90% of the total. γ-globulin was the only serum subfraction in which polysaccharide and protein curves paralleled each other.

Discussion. The current study has demonstrated that serum protein and glycoprotein changes previously observed in chronic



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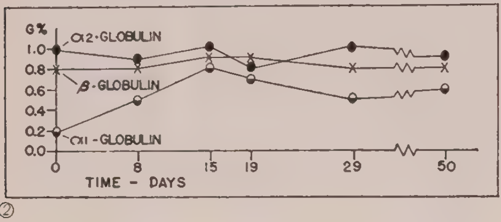

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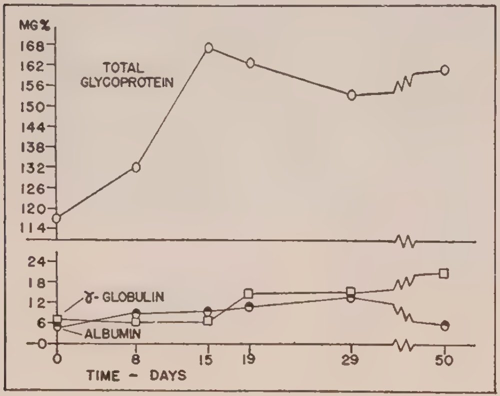

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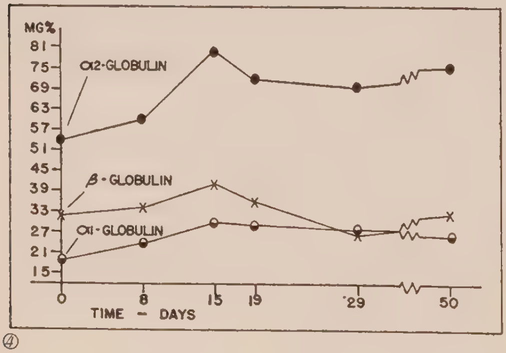

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FIG. 1 and 2. Alterations in serum protein patterns in early experimental tuberculosis.

FIG. 3 and 4. Alterations in serum protein-bound carbohydrate patterns in early experimental tuberculosis.

○, Total
◓, Albumin
◒, α_1-globulin
●, α_2-globulin
×, β-globulin
□, γ-globulin

infections occur quite early in the course of the disease. With the exception of increases in the components of the γ-globulin fraction, major alterations in other subfractions of serum had occurred 15 days following infection. The lack of further changes, notwithstanding a progressive increase in disease, strongly suggests that the observations may reflect primarily systemic manifestations of the inflammatory response. The hyperproteinemia previously noted in well-established experimental disease(8) was found to occur approximately 2 months following infection and was due to increase in γ-globulin levels. In contrast to the findings in clinical tuberculosis(1,2), the protein components of the α_2-globulin fraction were little affected by the disease.

Summary. The systemic effects of acute experimental tuberculosis as reflected by alterations in concentration and distribution of the proteins and protein-bound carbohydrates of serum were investigated by filter paper electrophoresis. Electrophoretic analyses were performed on serum samples from groups of adult female guinea pigs sacrificed 8, 15, 19, 29, and 50 days following inoculation with 0.1 mg *M. tuberculosis.* Significant increases occurred in total serum glycoprotein levels and in the protein component of the α_1-globulin fraction 8 days post-infection. A marked elevation of the polysaccharide moiety of the α_2-globulin fraction and a significant decline in albumin protein was observed 15 days following infection. With the exception of significant increases in the components of the γ-globulin fraction in the latter stages of the study, major alterations in the other subfractions of serum had occurred by 15 days post-infection. It is suggested that the early alterations in electrophoretic patterns represent primarily systemic manifestations of the inflammatory response.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Some Factors which Influence the Catalytic Activity of Pancreatic Cholesterol Esterase. (26091)

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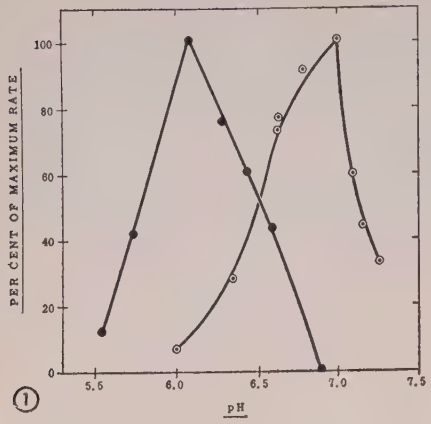
Extracts of beef pancreas catalyze a rapid synthesis and hydrolysis of cholesteryl oleate (1,2,3). Differences in some of the requirements for optimal activity of the two reactions have been noted(3). The influence of pH, electrolyte concentration, and β-sitosterol upon catalytic activity of the enzyme is considered further in the present report.

Methods and materials. Preparation of substrates and enzyme and procedure for manometric estimation of cholesterol esterase activity have been described(1). Briefly, the composition of the standard test substrate for synthesis was 0.04 M cholesterol or other sterol, 0.12 M lithium oleate, and 0.02 M cholic acid; for hydrolysis it was 0.07 M cholesteryl oleate, 0.014 M cholic acid, and 0.035 M lithium oleate. The reaction mixture contained substrate, $NaHCO_3$, and $(NH_4)_2SO_4$ in amounts indicated with the data of each experiment. The enzyme was a 10% aqueous or 0.01 M NaCl extract of acetone-dried beef pancreas clarified by filtration. The protein content ranged from 15 to 20 mg per ml(4). Approximately 0.3 ml was used in each reaction vessel. The reaction was conducted in conventional Warburg flasks at 37°C. After appropriate periods of gassing with CO_2 and temperature equilibration, the reaction was started by pouring the enzyme solution from the side-arm into the main compartment. The first reading was made 3 minutes later, to allow for non-specific gas exchange, and thereafter at desired intervals. An increase in the pressure of CO_2 occurs during ester hydrolysis; this is indicated by a plus sign. The pressure of CO_2 decreases during ester synthesis; this is shown by a minus sign. Results are reported either as relative activities or as μl CO_2 exchanged, *i.e.* released (+) or absorbed (–), per unit time.

Results. The effect of pH on rate of synthesis and hydrolysis of cholesteryl oleate at the concentration of electrolyte which is optimal for each reaction is seen in Fig. 1. It is evident that the optima are distinct and that at the optimal pH for each reaction the opposite reaction would not occur or its rate would be negligible. At intermediate values of pH both reactions take place although at reduced rates. Nearly 50% of the maximal rate at optimal pH occurs in the vicinity of pH 6.5. This pH is taken to be the single optimum for both reactions.

The data of Fig. 2 show the effect of electrolyte concentration at optimal pH for each reaction. It is seen that the concentration of electrolyte which is optimal for synthesis is inadequate for hydrolysis. Addition of electrolyte even in amounts slightly greater than required for optimal synthetic activity causes immediate precipitation of the substrate for synthesis, resulting in diminished reaction rate. Extent of decline in rate depends upon completeness of precipitation,



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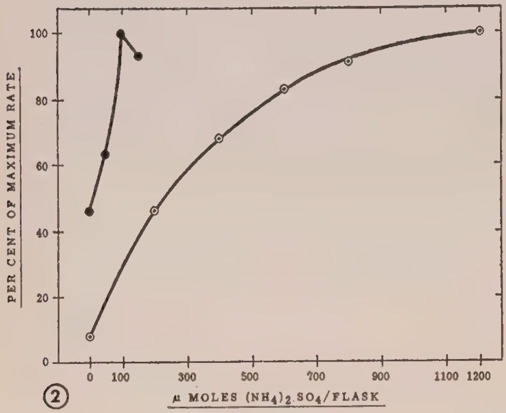

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FIG. 1. Activity as a function of pH. The standard test substrates for synthesis (●) and hydrolysis (○) of cholesteryl oleate were prepared at approximately the pH values indicated. Test systems consisted of 0.8 ml of the appropriate standard test substrate with 0.045M-$(NH_4)_2SO_4$ for synthesis and 0.72M-$(NH_4)_2SO_4$ for hydrolysis. $NaHCO_3$ concentration was altered in accordance with the Henderson-Hasselbach equation to obtain the various pH values. Appropriate amounts of NaCl were added to all reaction mixtures containing less than the maximum amount of $NaHCO_3$ used in the system to maintain a constant uni-univalent electrolyte concentration. Total reaction volume 2.2 ml; gas, CO_2. The pH values recorded are those found after the 30-min. experimental period, but do not differ from initial pH values by more than 0.1 pH unit.

FIG. 2. Effect of electrolyte concentration on rate of synthesis (●) and hydrolysis (○) of cholesteryl oleate. Each reaction mixture consisted of 0.8 ml of appropriate standard test substrate. Concentration of $NaHCO_3$ was 0.014M in the system for synthesis and 0.18M in that for hydrolysis; gas, CO_2. Amount of $(NH_4)_2SO_4$ was varied over the range shown on abscissa. Total reaction vol, 2.2 ml.

TABLE I. Selective Effect of Electrolyte Concentration. Standard test substrate for synthesis of cholesteryl oleate was used in Exp. 1. For Exp. 2 this was modified by addition of cholesteryl oleate equivalent in amount to cholesterol. For Exp. 3 cholesteryl oleate was substituted for cholesterol. Each reaction flask contained 0.8 ml of one of the substrates in a total fluid vol of 2.2 ml. Reaction mixture was maintained at pH 6.5-6.6 by 0.047M $NaHCO_3$ and CO_2. Minus and plus signs indicate ester synthesis and hydrolysis respectively.

		μl CO_2/60′		
No.	μmoles $(NH_4)_2SO_4$:	0	100	1200
1.	Cholesterol	−86	−107	−22
2.	" + cholesteryl oleate	−30	−18	+38
3.	Cholesteryl oleate	+58	+89	+127

The effect of electrolyte over a wide range of concentrations can be evaluated at pH 6.5-6.6. At this pH the dispersed character of the substrate for synthesis is not destroyed even by the high electrolyte concentrations required in the hydrolytic system. The data are given on Table I. At pH 6.5-6.6 the response of the two systems in presence of either substrate alone (Nos. 1 and 3) is qualitatively similar to that seen at the optimal pH for each reaction (Fig. 2). In the presence of both substrates a complete reversal of gas pressure change from negative (synthesis) to positive (hydrolysis) is brought about by increase of electrolyte concentration from 0 or 100 μmoles $(NH_4)_2SO_4$. to 1200 μmoles $(NH_4)_2SO_4$.

Both cholesterol and β-sitosterol are esterified with oleic acid although the optimal conditions are not the same(3). The data of Table II show that the percent of $4C^{14}$-cholesterol which is esterified decreases as the absolute amount of β-sitosterol is increased.

Discussion. Although the pH optima for synthesis and hydrolysis of cholesteryl oleate differ by approximately one pH unit, it has been shown that both reactions can occur at a single intermediate pH. In the presence of both substrates the reaction which predominates at this pH, as determined by the direction of gas exchange, is governed by the electrolyte concentration. At low concentrations of electrolyte the esterification reaction is favored, whereas at high concentrations of electrolyte hydrolysis is dominant. These results are in accord with the effect of electro-

TABLE II. Effect of β-Sitosterol upon Rate of Esterification of Cholesterol. 0.1 ml of the standard substrate for synthesis of $4C^{14}$ cholesteryl oleate was present in all flasks. A similar substrate, but prepared with β-sitosterol, was added in amounts to give the ratios indicated; fluid vol, 2.2 ml. Concentration of $(NH_4)_2SO_4$ was 0.023M and of $NaHCO_3$, 0.014M; gas CO_2. Reaction mixture was extracted with 1:1 acetone:alcohol (v/v) and free sterol was removed as the digitonide. Activity of the supernate, combined with washings of the digitonide, was determined by a Packard Tri-Carb Scintillation Counter.

Ratio, cholesterol/β-sitosterol	% $4C^{14}$-cholesterol in ester
1:0	60.0
1:1	41.9
1:2	37.5
1:4	23.4
1:6	21.9
1:8	15.0
1:10	13.0

lyte concentration on reaction rate with each substrate alone.

The magnitude of gas exchange in the presence of both substrates does not appear to be related in any obvious way to the magnitude of change observed with each substrate alone. At low electrolyte concentrations the rate in the presence of both substrates is approximately equal to the algebraic sum of the individual rates. If it can be assumed that the concentration of each reactant when both are present is sufficient to saturate the enzyme, as is the case when each is used alone, then the data may suggest that a unique catalytic site is required for synthesis and hydrolysis. At high electrolyte concentration summation of activity does not occur; hence it would appear that the hydrolytic reaction is inhibited by cholesterol. It may be that only at high electrolyte concentration cholesterol alters the colloidal properties of the substrate for hydrolysis making it less susceptible to enzymic attack.

The inhibitory effect of β-sitosterol upon intestinal absorption of cholesterol has been amply documented(5). β-Sitosterol is not absorbed in significant amounts(5) although it is readily esterified *in vitro*(3,5) and presumably *in vivo*. Thus it can be anticipated that rate of cholesterol esterification will be influenced by amount of β-sitosterol present, as is verified by the data. The function of pancreatic cholesterol esterase is associated with the process of cholesterol absorption(5). Interference with esterification of cholesterol by an esterifiable but non-absorbable sterol may be one important mode of action of β-sitosterol.

It is suggested that conflicting data on the type of reaction catalyzed by pancreatic cholesterol esterase may be attributed to insufficient regard for the factors which have been shown to influence the catalytic properties of this enzyme.

Summary. These data indicate that pancreatic cholesterol esterase catalyzes both synthesis and hydrolysis of cholesteryl oleate at a single pH, but the reaction which predominates in presence of both substrates is governed by the electrolyte concentration. Rate of synthesis of $4C^{14}$-cholesteryl oleate is decreased in proportion to relative concentration of β-sitosterol.

The authors acknowledge with gratitude the continued interest of Drs. R. E. Shipley and C. W. Pettinga.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Relative Susceptibility of Young Mice and Hamsters to Coxsackie B-3 Virus. (26092)

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Mice and hamsters are considered to be the experimental animals of choice for study of Coxsackie viruses. Much information has been published on the effects of these viruses on suckling mice. It is well known that for most strains of Coxsackie viruses the susceptibility of the mouse is inversely related to the age of the animal. Thus for isolation of these viruses from clinical specimens it is recommended that mice be less than 24 hours old(1).

The utility of the hamster in studies on Coxsackie viruses has received little attention. The purpose of the study reported here was to compare age susceptibility of suckling hamsters with that of mice to a strain of Coxsackie Group B-3 virus.

Methods. Virus. The 4560 strain of Coxsackie B-3 virus was obtained from Dr. R. H. Heubner of the N. I. H. with a history of 9 passages in mice. A stock pool of a 10% virus suspension in physiological saline was prepared in the usual manner by injecting suckling mice intraperitoneally, harvesting and processing carcasses at the time animals were moribund. Virus was stored in a dry-ice chest. Aliquots of this pool which represent 10th serial passage virus in mice were used for all titrations in Experiment I. Another pool was prepared about a year later and this 11th passage virus was employed in all titrations in Exp. II. *Mice and hamsters.* The ICR strain of mice was used throughout. It had been previously determined that the susceptibility of these mice to the above strain of virus was about the same as that of CFW mice. Pregnant hamsters were obtained from Lakeview Hamster Colony, Newfield, N. J. and allowed to parturate in our laboratories. Ages of all suckling animals were accurate to within ± 15 hours. *Experimental procedure.* Age susceptibility of the hamsters and mice was compared by titrating stock virus in the various age groups of both species. At the time of titration animals of a given age were randomized by pooling and redistributing them back to dams. Usually at least 8 animals per dilution of virus were inoculated, but in a few titrations 2 unrandomized litters per dilution were used. Groups of hamsters and mice were injected intraperitoneally with 0.1 ml amounts of the various dilutions of virus. Titrations were scheduled so that several different age groups of each species were tested on the same day. A titration in younger animals was included each day to ascertain that maximum infectivity of the stock virus sample had been retained. Following inoculation animals were examined daily for 21 days. Criteria for infection were death or signs of neurological disturbance. Hamsters once developing signs usually succumbed. A few mice in the "older" age groups showed only mild signs followed by recovery. Titers are thus expressed as 50% infectious doses, but since most sick animals eventually succumbed, the infectious dose approximated the lethal dose.

Results. Both hamsters and mice showed essentially similar clinical responses to the infection. Few signs were noted prior to death of young animals receiving higher concentrations of virus. Young animals receiving higher dilutions of virus and older animals exhibited various manifestations of central nervous involvement including tremors, incoordination, hyperaesthesia in the early stages followed by depression, paralysis, coma and death. A few animals, especially mice, showed front leg paralysis involving one or both limbs. In hamsters the hind quarters were frequently paralyzed.

Incubation periods were shorter in the hamster. Day-old animals receiving 10% virus suspension showed first signs 24 hours following injection while day-old mice similarly inoculated appeared normal until the 3rd or 4th day after inoculation.

Table I, which presents typical titrations

TABLE I. Titration of Coxsackie B-3 Virus in Mice and Hamsters.

	Virus log dilution	–1	–2	–3	–4	–5	–6	–7	–8
Mice—	No. infected*	8	8	8	5	0			
	Incubation period (days)	4	5	7	8	—			
Hamsters—	No. infected*	8	8	8	8	8	7	4	1
	Incubation period (days)	1	2	2	3	5	7	7	8

* Eight animals were inoculated with each dilution of virus.

in each species, illustrates the differences in incubation periods. Also shown is the greater sensitivity of the hamster. The titer in the 3 day-old hamster was $10^{-7.0}$ compared to $10^{-4.1}$ obtained in the 2-day-old mouse.

Age-susceptibility comparisons are shown graphically in Fig. 1. The straight lines are drawn according to the method of least squares. Exp. I was completed about a year before Exp. II. Both experiments show the greater sensitivity of the hamster. Mice became refractory when they reached approximately one week of age. In hamsters of the same age the virus titer was still quite high. Hamsters became refractory at about 12 days.

Discussion. The indicator for infection in these studies was clinical response. It is probable that the susceptible period would be extended if subclinical infection were measured as shown in studies by Howes(2).

The strain of mice employed in these studies was possibly relatively resistant to the virus. However, in a few preliminary tests, the CFW strain of mice appeared to be of about equal susceptibility. Thus, it seems important to note that the hamster was the more susceptible species despite the fact that the strain of virus was adapted to the mouse and furthermore, that the inoculum employed was grown in mice.

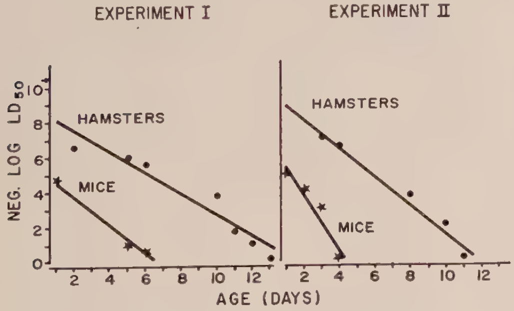

FIG. 1. Age-susceptibility of young hamsters and mice to Coxsackie B-3 virus. Points indicate infectivity titer of stock virus in the 2 species as influenced by age.

Although the present report considers only one type of Coxsackie virus, it suggests that similar comparisons be made with other types. In several preliminary titrations, it was noted that the hamster was more susceptible than the mouse to Coxsackie B-2 virus (Powers strain). For example, the titer in day-old mice for this virus was $10^{-6.0}$, while in 10-day-old hamsters a value of $10^{-8.3}$ was obtained.

Although tissue cultures are commonly employed by diagnostic laboratories for isolation of viruses, not all Coxsackie virsues grow in cell cultures. It thus becomes necessary to test specimens in experimental animals. Most laboratories using mice for diagnostic purposes experience difficulty in having a supply of newly-born mice on hand. If the present observations hold true for other Coxsackie viruses, the mouse problem would be obviated. Day-old hamsters could be obtained at the first of the week and used throughout the week. The increased period of susceptibility of the hamster might also be useful in another way. It has been found that hamsters injected at 5 days of age with inactivated preparations of 5 different types of Coxsackie B viruses were resistant to challenge at 10 days with virulent homologous strains but were susceptible to heterologous types. This suggests that a challenge test could be developed for typing Coxsackie viruses.

Summary. Hamsters were more susceptible than mice to a strain of Coxsackie B-3 virus. This was shown by comparing the response of these species to virus as influenced by age. At a given age a stock virus preparation showed a consistently higher titer in the hamster. Furthermore, the period of suscep-

tibility of hamsters was about twice that of mice.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Effect of Intravenous Injection of Oxidized Methyl Esters of Unsaturated Fatty Acids on Chick Encephalomalacia.* (26093)

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Diets high in unsaturated fats and deficient in Vit. E are known to cause chick encephalomalacia(1,2,3) which can be prevented by addition to the diet of tocopherols or specific synthetic antioxidants(4,5,6) but not by selenium(7,8). As autoxidation of unsaturated fats can readily occur in an environment devoid of antioxidants, encephalomalacia may represent the consequence of consuming peroxidized fat or may represent the result of peroxidation *in vivo*. Furthermore, it has recently been observed that inclusion of various oxidized fatty acids(9) or fat(10) in the diet accelerates the incidence of the disease. The present study was designed to clarify the effect of oxidized fatty acids on development of encephalomalacia.

Methods. To compare the effect of various oxidized fatty acids on incidence of cerebellar disorders, 4 groups of 4 to 8 one-week-old male chicks (New Hampshire Columbian cross) were fed *ad libitum* for 2 weeks a purified diet(9) which contained 10% corn oil‡ and 4 groups the same diet supplemented with 8 mg % DL-α-tocopherol. The diets were prepared daily. The oxidized fatty acids consisted of the hydroperoxide of methyl linoleate(11), the reduced hydroperoxide of methyl linoleate(12), and methyl 12-oxo-*cis*-9-octadecenoate prepared from methyl ricinoleate(13). These preparations were emulsified in chick serum (10 mg/ml) at approximately 1000 rpm for 1 minute at 0°C with a Teflon pestle homogenizer and 1 ml injected intravenously. After injection, the birds were allowed access only to water. When symptoms of cerebellar disorders were observed, the birds were sacrificed for histological examination(9).

In another study, week-old chicks were divided into 12 groups of 6 to 12 birds each and fed *ad libitum* Vit. E deficient diets. These diets contained 35.30 or 11.77% soy (ADM) protein, with or without inositol, ascorbic acid and varied amounts of stripped corn oil§ substituted into the diet at the expense of glucose. The diets were prepared daily. Birds which had not shown symptoms of encephalomalacia after 1 week were injected intravenously with 10 mg of methyl linoleate hydroperoxide emulsified in 1 ml of serum. The effect of dietary pretreatment on the severity of cerebellar disorders induced by intravenous injection of the lipohydroperoxide could thus be compared.

Four groups of 16 chicks which had been kept on the high protein, Vit. E deficient diet (treatment 2) were also injected as follows: one, 1 ml of low density lipoproteins (S_f 20-400 class) obtained from chick serum(14); two, 1 ml of the low density lipoproteins which had been denatured by heating for 1 minute at 100°C; three, 1 ml emulsified methyl linoleate hydroperoxide (10 mg/ml serum); and four, 10 mg linoleic acid hydro-

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* Supported by research grant from Nat. Inst. of Health, U.S.P.H.S., Dept. of H.E.W.

† On leave of absence, Nagasaki Univ. School of Med., Nagasaki, Japan.

‡ Mazola, furnished through courtesy of Corn Products Refining Co., Argo, Ill.

§ Corn oil stripped of vit. E by high vacuum distillation, furnished through courtesy of Distillation Products, Inc., Rochester, N. Y.

TABLE I. Effect of Intravenous Injection of Oxidized Methyl Esters of Unsaturated C_{18} Fatty Acids on Cerebellar Disorders.

		Cerebellar disorders	
		Total chicks	
Group	Lipid inj.	No supplement	8 mg % tocopherol
1	Methyl linoleate hydroperoxide	5/8	0/8
2	Reduced methyl linoleate hydroperoxide	0/4	0/4
3	Methyl 12-oxo-*cis*-9-octadecenoate	0/4	0/4
4	Methyl linoleate	0/4	0/4

peroxide(15) in 1 ml serum.

Results. Intravenous injection of as little as 10 mg of the hydroperoxide of methyl linoleate into chicks kept on a diet which contained 10% corn oil induced cerebellar disorders such as ataxia, tremors, and retropulsive movement 1-5 hours after injection (Table I). No cerebellar disorders were noted after injection in birds kept on the diet which had been supplemented with 8 mg % α-tocopherol. Injection of reduced hydroperoxide, methyl 12-oxo-*cis*-9-octadecenoate, or fresh methyl linoleate caused no cerebellar disorders. Thus, of the oxidized fatty acids tested, only lipoperoxide initiated symptoms of cerebellar disorders. Pathological examination of tissue of the cerebellum indicated that injection of methyl linoleate hydroperoxide caused considerable cerebellar edema or congestion of the capillaries. No detectable necrosis or hemorrhage, which has been considered to accompany the so-called nutritional encephalomalacia, was noted.

The presence of the mixed tocopherols in corn oil(16) might have partially prevented destruction of the cerebellum, since 8 mg % of additional α-tocopherol completely prevented the cerebellar disorders caused by injection of methyl linoleate hydroperoxide. That the mixed tocopherols naturally present in corn oil influenced the development of cerebellar disorders was shown in chicks kept on diets which contained corn oil stripped of Vit. E. Response to injection of lipohydroperoxide into chicks kept on such Vit. E deficient diets was found to be greatly influenced by dietary composition.

Diets which caused a high incidence of nutritional encephalomalacia also caused severe destruction of the cerebellar tissue after injection of methyl linoleate hydroperoxide. A high dietary protein level greatly accelerated the rate of incidence of nutritional encephalomalacia (Table II). Furthermore, rate of incidence depended on the level of

TABLE II. Effects of Composition of Diets on Chick Nutritional Encephalomalacia and on Cerebellar Disorders Caused by Injection of Methyl Linoleate Hydroperoxide.*

								Effect of intrav. inj. of methyl linoleate hydroperoxide in survivors			
Treatment No.	Soy (ADM) protein, %	Stripped corn oil, %	Ascorbic acid, mg %	Inositol, mg %	Feed intake 7 days, g	Wt gain 7 days, g	No. chicks with encephalomalacia	Total No. chicks	No. chicks with cerebellar disorders	Chicks showing hemorrhage and necrosis No.	%‡
1	35.30	2	0	0	146	96	0/12	6	4	1	25
2	35.30	10	0	0	142	96	5/12	6	6 (2)†	6 (2)	100
3	35.30	20	0	0	140	59	10/12	2	2	2	100
4	11.77	2	0	0	144	52	0/12	6	1	0	0
5	11.77	10	0	0	129	45	0/12	6	5	1	20
6	11.77	20	0	0	129	46	2/12	6	4	2	50
7	35.30	10	75	0	136	96	2/6	4	4 (2)	0	0
8	35.30	10	0	30	141	82	4/6	2	2 (2)	2 (2)	100
9	35.30	10	75	30	129	89	0/6	6	6 (2)	1	17
10	11.77	10	75	0	129	51	0/6	6	5	0	0
11	11.77	10	0	30	129	47	0/6	6	6 (1)	0	0
12	11.77	10	75	30	133	49	0/6	6	5	0	0

* 0.30 or 0.10% of glycine was added to high and low protein diet(9), respectively. Supplements to basal diet were made at expense of glucose on weight basis. † ( ) indicates shock-like symptoms developed in chicks. ‡ Out of No. which showed cerebellar disorders.

stripped corn oil; a high fat, high protein diet resulted in the highest incidence of encephalomalacia. Although the presence of either ascorbic acid or inositol alone was not effective in preventing a high incidence, the presence of both ascorbic acid and inositol depressed the incidence of encephalomalacia.

When the chicks which had not developed symptoms of encephalomalacia were injected with emulsions of methyl linoleate hydroperoxide, all of the chicks which had been kept on diets high in protein and fat (treatments 2, 3, 8) showed cerebellar ataxia and various degrees of degeneration, necrosis and hemorrhage of the cerebellar tissue after injection. Engorged capillaries were scattered through the necrotic tissue and sometimes accompanied an increase of the glia cells. No striking sign of organization in the malacic area was noted. These histological findings could be considered to represent an acute and a relatively early stage of the cerebellar lesion. Injection of methyl linoleate hydroperoxide did not cause lesions in the cerebrum, optic lobes, basal ganglia, medulla, or spinal cord. Most of the chicks, in treatments other than 2, 3, 6, or 8 which showed cerebellar disorders after injection of methyl linoleate hydroperoxide, revealed only dilation of the capillaries or cerebellar edema with disruption and separation of the cellular and fibrillar elements.

We noted that unless chicks showed positive symptoms of cerebellar disorders, no noticeable cerebellar lesions were present. Furthermore, symptoms of encephalomalacia were not precipitated by removal of feed from the chicks. Cerebellar disorders and the subsequent cerebellar lesions appeared to be induced only as a result of injection of methyl linoleate hydroperoxide. Although the emulsion of methyl linoleate hydroperoxide was injected slowly, some chicks died immediately in shock. Autopsy of these chicks indicated that only chicks which had been kept on a diet high in protein and fat (treatments 2, 8) showed noticeable cerebellar necrosis or hemorrhage. Chicks which had been supplemented with Vit. E did not go into shock even after rapid injection of methyl linoleate hydroperoxide. It therefore appeared that physiological conditions brought on by dietary pretreatment predisposed chicks toward shock.

As it has previously been reported that methyl linoleate hydroperoxide selectively or preferentially denatures serum β-lipoproteins *in vitro*(11), it was possible that denatured β-lipoproteins in the emulsion of methyl linoleate hydroperoxide might have caused cerebellar disorders after injection. However, injection of a 1 ml solution of heat denatured lipoprotein did not cause cerebellar disorders or shock. Thus, the initiation of cerebellar disorders appeared to be solely due to injection of the hydroperoxide of methyl linoleate. The hydroperoxide of linoleic acid was as effective as the hydroperoxide of methyl linoleate.

Discussion. Cerebellar disorders can be developed in chicks by 2 different but related means: (1) by injection of lipohydroperoxide and (2), by feeding of a diet high in unsaturated fat and deficient in Vit. E. The extent of cerebellar damage seemed to depend on dietary level of Vit. E, protein, fat, inositol, ascorbic acid and possibly other dietary factors.

The emulsion of methyl linoleate hydroperoxide may have decomposed after injection or other oxidative changes in the hydroperoxide may have occurred with time. However, the fact that injected reduced methyl linoleate hydroperoxide or a long chain carbonyl compound, methyl 12-oxo-*cis*-9-octadecenoate did not induce encephalomalacia seems to suggest that lipohydroperoxide, not the oxidized products derived from the fatty acids *in vivo,* is the initiating agent in encephalomalacia.

Nutritional encephalomalacia may develop as a result of both functional and organic disturbances in cerebellar tissue by lipohydroperoxide. When a sufficient but still immeasurable level of lipohydroperoxides has accumulated in the blood or some tissue site, they may act preferentially on the circulatory system in the cerebellum. Although 10 mg of hydroperoxide was sufficient to precipitate cerebellar disorders when injected, less lipohydroperoxide may be sufficient to induce nutritional encephalomalacia. It is possible that a sufficient amount of lipohydroperoxide may accumulate from dietary sources in the blood of Vit. E deficient chicks to induce encephalomalacia, although most of the lipohydroperoxide may be destroyed during intestinal absorption. Nutritional encephalomalacia may also be accentuated by dietary prerequisites, such as Vit. E deficiency, a high fat, high dietary protein level, and absence of dietary ascorbic acid and inositol.

We have previously shown that methyl linoleate hydroperoxide inhibits lipid absorption in rats(11). However, when diluted with fresh methyl linoleate, some lipohydroperoxide seemed to be absorbed. Furthermore, the amount of lipohydroperoxide absorbed may not necessarily be proportional to the peroxide content of ingested fat. It is also possible that during Vit. E deficiency unsaturated fatty acids in serum and tissue lipoproteins may be peroxidized *in vivo* to give an additional amount of lipohydroperoxides to induce encephalomalacia.

It has recently been shown that the low density lipoproteins contain a major part of total serum tocopherol(17). Furthermore, we have recently shown that low density lipoproteins are very easily peroxidized *in vitro* and that hematin compounds greatly accelerate formation of lipohydroperoxide in isolated low density lipoproteins which resulted in rapid denaturation of the lipoproteins(18). Therefore, diets deficient in Vit. E, or containing high amounts of unsaturated fatty acids may not only be more susceptible to peroxidation, but may also favor *in vivo* peroxidation of serum low density lipoproteins. The aggravating effect of oxidized fatty acids or heated oils on encephalomalacia may be due to 1) their activity as prooxidants, 2) their peroxidation and destruction of Vit. E in the diet, and 3) their ability to cause lipoproteins to be more susceptible toward peroxidation *in vivo*.

Summary. Cerebellar disorders developed in chicks 1-5 hours after intravenous injection of 10 mg of the hydroperoxide of methyl linoleate or of linoleic acid emulsified in 1 ml of serum. No cerebellar disorders were noted, after injection, in birds kept on an identical diet but supplemented with 8 mg % α-tocopherol. Injection of reduced hydroperoxide, methyl 12-oxo-*cis*-9-octadecenoate or fresh methyl linoleate caused no cerebellar disorders indicating only lipohydroperoxide initiated symptoms. The severity of cerebellar disorders induced by injection of lipohydroperoxide was greatly influenced by dietary pretreatment. The cerebellum of chicks which had been kept on high protein, high fat, Vit. E deficient diets was damaged more extensively by injection of lipohydroperoxide than the cerebellum of chicks kept on adequate diets. These data suggest that nutritional encephalomalacia may be initiated by accumulation of sufficient, though still immeasurable, levels of lipohydroperoxide *in vivo*.

The authors wish to thank D. R. Black, M. F. Kummerow, Sandra J. Curtis and Valerie J. Hall for technical assistance.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

A Solar Daily Variation in Oxygen Consumption of the Embryonated Egg.*
(26094)

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For a number of years this laboratory has pursued an extensive comparative study of the persistent metabolic rhythms in algae, angiosperms, invertebrates, and vertebrates. These metabolic rhythms possess basic, component, solar and lunar daily variations which predominate or underlie the phase-labile, adaptive rhythms of special processes when the latter are present. The occurrence of such variations is called extrinsic rhythmicality because the periods, and at least to some extent, the amplitudes and forms, are derived in direct response to some subtle geophysical rhythm(1). The disclosure of these variations in all organisms studied suggests that this is a universal phenomenon. If there were present such fluctuations in the metabolic state of the embryonated egg, they might constitute a variable whose importance has not been recognized to date. Therefore, the following study was undertaken to learn (i) if systematic fluctuations did indeed occur and (ii) something of their character were this the case.

Materials and methods. O_2-consumption of individual eggs was measured with a Brown automatic recording respirometer(2, 3) in constant illumination, temperature, humidity, pressure, CO_2- and O_2-tension, etc. It was necessary to modify the respirometer chamber somewhat. A glass tumbler having a volume of 110 cc was fitted with a No. 11 rubber stopper. To the upper end of this stopper was sealed a plastic (Saran) sack of about 60 cc capacity which was the oxygen reservoir. The rubber stopper was penetrated by a 26-gauge hypodermic needle which served as a capillary connection between the collapsible oxygen reservoir and the chamber beneath. In operation 10 cc of water, a vial of KOH solution, and a small glass frame were placed in the bottom of the tumbler. The egg was rested air sack uppermost on the 4 tips of the glass support. The flask was stoppered, leaded until it just submerged, and suspended beneath the recorder in the water bath of a barostat at 38 ± .05° C. The unit was hermetically sealed and aspirated to a constant pressure of 28.7 in. Hg.

Eggs were obtained periodically from a local commercial source between Nov. 1959, and April, 1960. Before its oxygen consumption was recorded, each egg was allowed to warm to room temperature and was then placed in a forced-draft incubator at 38°C at about 4 P.M. C.S.T. The day on which incubation was initiated was designated Day 1. During the morning or afternoon of Day 2 or 3 the egg was transferred to the respirometer in which incubation was continued until termination of the experiment on Day 7. The egg was then opened and inspected. If an embryo did not appear to have developed normally its record was discarded. Ordinarily the oxygen supply was depleted on Day 7. Some data for Day 8 were obtained by opening the barostat for a 10-minute interval on Day 7 and replenishing the supply of oxygen and of CO_2-absorbent. This study is restricted to a 4-day period in development. The onset of the period is determined by the sensitivity of the recorder and the end, by the capacity of the O_2-reservoir.

Results. About 1800 hours of respiration

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* Aided by contract between Office of Naval Research, Dept. of Navy, and Northwestern Univ.

TABLE I. Rates of Oxygen Consumption for Normal Embryonated Eggs as Determined by Various Investigators.

Hr of incubation	This study	Romanoff (4)	McLimans *et al.* (5) Series A‡	McLimans *et al.* (5) Series B‡	Murray (6)*	Hasselbalch (7)†	Greiff *et al.* (8) Spring setting
72	.226 ±.066§ (6)‖	.253 ±.040 (4)					
96	.427 ±.110 (25)	.381 ±.100 (6)	.34 ±.11 (12)				
120	.680 ±.131 (26)	.524 ±.052 (6)	.44 ±.05 (25)			.63	.61
144	.826 ±.178 (9)	.831 ±.134 (9)	.89 ±.07 (11)	.91 ±.06 (3)	.88	.92	1.54
168	1.423 ±.191 (14)	1.421 ±.223 (6)		1.19 ±.14 (6)	1.32	1.77	2.08
192	1.922 ±.173 (4)	1.671 ±.173 (4)	1.23 ±.26 (33)	1.51 ±.03 (5)	1.99	2.43	2.26

Rate as cc of O_2/egg/hr. * Calculation of Needham (9) from Murray's data. † As cited by McLimans *et al.* ‡ Series of different genetic patterns. § Stand. dev. ‖ No. of observed eggs.

data were collected and distributed as follows, 24 complete Day 5's, 23 complete Day 6's, 8 complete Day 8's, and several incomplete days from slightly earlier and later stages. The validity of results obtained under the conditions of this experiment was tested by comparing them with results of other investigators who have employed a variety of technics to measure O_2-consumption in the intact egg (Table I). These data are in such consistently good agreement over the 6-day period that one may safely conclude the Brown recording respirometer to be a reliable instrument for purposes of this study.

To disclose any daily pattern which might be superimposed upon the sigmoid curve characteristic of O_2-consumption during development, the data were reduced as follows. A straight line was constructed arbitrarily between the average 1-2 A.M. value and the average 11-12 P.M. value for each complete day of data. The deviation of each hourly reading from this linear daily trend-line was measured. In Fig. 1 are depicted the mean daily patterns of O_2-consumption for 3 days of incubation obtained as mean hourly deviations from daily trend. The mean linear trend expressed in cc/egg/hr is from 0.226 to 0.497 on Day 5, 0.518 to 0.747 on Day 6, and 1.186 to 1.508 on Day 8. It is readily apparent that the 3 days possess a common diurnal pattern in average hourly rate of O_2-consumption. Through the morning hours rates are characteristically low but rise in the afternoon to a sharply defined maximum at 7 P.M. and then return rapidly to the trend-line. This pattern is indicated by the following statistically significant differences between means of maximal and minimal hourly rates: 5 A.M. and 7 P.M. on Day 5, $P < .001$; 5-6-7 A.M. and 6-7-8 P.M. on Day

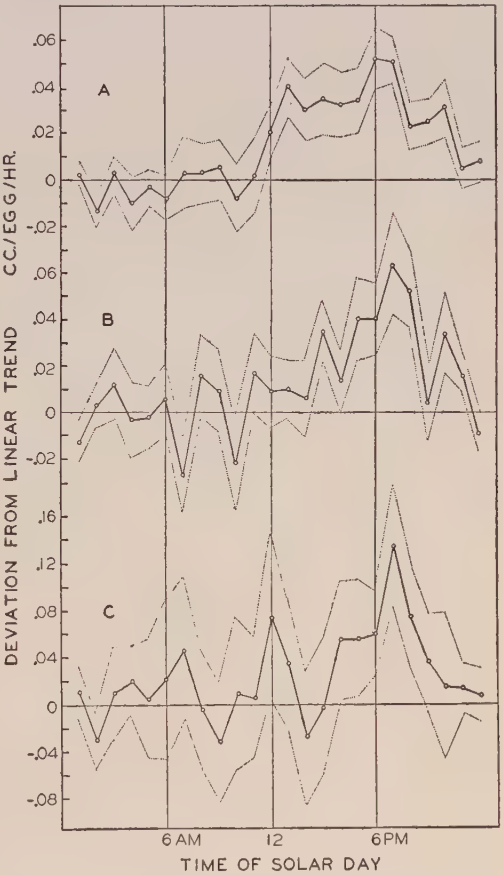

FIG. 1. Daily variations in O_2-consumption for 3 days of incubation, as mean hourly deviations from daily trend. A. Day 5, avg of 24 eggs. B. Day 6, avg of 23 eggs. C. Day 8, avg of 8 eggs. Stand. errors for all hours are indicated. Time is CST.

6, $P<.001$; and 8-9 A.M. and 7-8 P.M. on Day 8, $P<.02$. The mean range of the fluctuation is 17.1% of the mean daily rate on Day 5, 13.0% on Day 6, and 12.3% on Day 8.

It will be recalled that all data were obtained while the recorder and organism were hermetically sealed in a large rigid container maintained at constant ambient pressure. During this study the barometric pressure was recorded with a microbarograph at the laboratory in Evanston. Analysis of the O_2-consumption data revealed that the 7 P.M. deviation from linear trend on Days 5 and 6 was correlated with the 2-6 P.M. rate of barometric pressure change on the same day within the limits −0.12 to +0.08 in. Hg ($r = +0.446$, $N = 41$, $P<.005$). Range in metabolic deviation was substantial, −0.12 cc/egg/hr to + 0.28 cc/egg/hr.

Discussion. It is well-known that the period of laboratory incubation cannot be used to determine embryonic stage of development. Among eggs incubated for the same period variation in stage may be considerable, particularly in the first 4 or 5 days of incubation(10). Individual or chance factors which contribute to this variability are genetic differences(11), differences in length of the latent period(10), uneven retention of the egg in the oviduct, and temperature of environment in storage and transport prior to incubation(12). In this study the investigator made no attempt to control these factors. Thus, recurrence of a sharply defined maximum in O_2-consumption at the same time on successive days is prima-facie evidence that the observed diurnal fluctuations are *not* correlated with any particular developmental process (morphogenesis, differentiation). The direct relationship of metabolic activity to time of solar day indicates that one is dealing with a protoplasmic response identical to manifestation of a persistent rhythm. The daily pattern in O_2-consumption appears to be independent of morphological development within limits of variation for embryos of the same incubational age. However, in that new levels of development are rapidly being achieved, it is conceivable that the form of daily fluctuations, other than the ones studied here, may be a function of the incubation period. Such a possibility is clearly suggested by diversities in minor components of the 3 mean fluctuations in Fig. 1.

The correlation of 7 P.M. metabolic activity with coincident 2-6 P.M. rate of barometric pressure change can be adduced as further support for identification of these diurnal fluctuations with extrinsic rhythmicality. Such correlations with this specific pressure parameter have been described for metabolic rhythms in organisms as diverse as the potato(3), fiddler crab(13), and mud snail (14). The existence of this relationship to atmospheric pressure change in an organism which so nearly approaches a closed system is obviously further evidence for the universal nature of this phenomenon. Not only does the metabolic state of the organism vary with time of day, but also from one day to the next, as it responds to its varying geophysical environment.

Summary. The O_2-consumption of individual embryonated eggs was measured continuously for 3 days in constant conditions, including pressure. Superimposed on linear daily trend for Days 5, 6, and 8 was a mean pattern in hourly rates possessing a characteristic morning minimum and 7 P.M. maximum. This 7 P.M. deviation from daily trend was related to the concurrent 2-6 P.M. rate of atmospheric pressure change. This indicates that the solar daily variations of oxidative metabolism in the intact egg are a manifestation of extrinsic rhythmicality.

The author gratefully acknowledges the valuable suggestions and criticisms of Dr. F. A. Brown, Jr.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Observations on the Role of Lipids in Serum-Induced Intravascular Thrombosis.* (26095)

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(Introduced by F. J. Stare)
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Thrombus formation in diseased coronary arteries is the most common clinically important complication of atherosclerosis. Moreover, it has been suggested that the atherosclerotic process itself may result from endothelialization of intravascular thrombi(1). Dietary and serum lipids have been shown to be associated with the incidence and prevalence of coronary heart disease in human populations. Whether lipid alterations can affect coagulability of blood leading to thrombosis, as well as possibly contributing to the development of the underlying atherosclerosis, is not known. Alterations of serum lipids can produce marked atherosclerosis in experimental animals, yet there are few reports of induced thrombosis in such experiments(2). To investigate the possible role of lipids in coagulability of blood, there have been many studies utilizing various *in vitro* technics. The controversial results have been well summarized(3,4).

Wessler(5) reported on a method of inducing intravascular thrombi in dogs in a venous segment isolated after systemic injection of homologous serum. A later communication(6) presented an extension of this *in vivo* technic to production of thrombi in rabbits following infusion of heterologous (human) serum. This communication also presented evidence to show that the active serum factors necessary in this system are Factor IX (Plasma Thromboplastic Component), Hageman Factor, and possibly Plasma Thromboplastin Antecedent(7).

In an effort to quantitate the dose-response-time interrelationships we have adapted Wessler's method to the use of chickens as the assay animals. Chickens are cheap, grow rapidly, and are easily maintained. Some preliminary findings of the effects of lipids on this system are presented.

Materials and methods. White Leghorn cockerels approximately 3 to 4 weeks of age (200 to 300 g) were used. Some of the birds were fed a commercial Chick Starter mash, others received purified diets containing 0.8% cholesterol in which the source of fat was varied. The composition of these has been described(8).

Pooled human serum lots were stored at 4°C and used within one week of collection. Pooled chicken serum from birds on the mash diets was similarly stored for use.

Cholesterol determinations on the assay birds' sera and on the infused test sera were performed by the method of Carpenter *et al.* (9).

Chickens were anesthetized with intraperitoneal sodium pentobarbital (Nembutal) and a 5 cm segment of non-branching jugular vein was exposed. The test serum was infused into the contra-lateral jugular vein and al-

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* Supported in part by grants-in-aid from Nat. Inst. Health, Nat. Heart Inst., P. H. S., Bethesda, Md.; John A. Hartford Memorial Fund; Albert and Mary Lasker Foundation, New York; Nutrition Foundation, New York; and the Fund for Research and Teaching, Dept. of Nutrition.

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TABLE I. Quantitative Assay of 2 Samples of Pooled Human Serum.

ml	No. of assays	10 min. Segments clotted No.	10 min. Segments clotted %	15 min. Segments clotted No.	15 min. Segments clotted %
Serum I					
1.0	13	1	7.7	1	7.7
1.5	13	1	7.7	2	15.4
2.0	13	0	0.0	6	46.1
2.5	13	4	30.8	10	76.9
3.0	13	8	61.5	12	92.3
Serum II					
1.0	12	0	0.0	0	0.0
1.5	12	1	8.3	2	16.7
2.0	12	0	0.0	3	25.0
2.5	12	2	16.7	6	50.0
3.0	12	3	25.0	7	58.3
3.5	12	5	41.7	10	83.3

50% endpoint: Serum I —2.067 ml ± .145 ml.
Serum II—2.579 ml ± .176 ml.
$p < .05$ and $> .02$

lowed to mix for one and one-half minutes at which time the exposed segment was isolated with rubber-tipped clamps so as to create 2 contiguous segments. At appropriate times the segments were opened *in situ* and the contents collected on filter paper. Results were reported as either the presence or absence of gross clot.

Results. Blood in isolated venous segments remained fluid for at least one hour when no serum infusion was given. Neither normal saline nor homologous (chicken) serum produced intravascular clots.

Using 10 and 15 minutes as the endpoints for opening the 2 venous segments, human serum was active in the dose range 1.5 to 3.5 ml per bird. Table I presents the findings in 2 lots of pooled human serum tested at different times on 2 groups of chickens raised on commercial mash diets. These dose response curves are plotted in Fig. 1. When compared by the Reed-Muench method(10) of establishing 50% endpoints (standard error determined by the method of Pizzi(11)), the difference between the 2 sera at 15 minutes is significant at the .05 level.

TABLE II. Clots Induced by 3.0 ml of a Pooled Human Serum Lot at a 15 Minute Endpoint.

Diet	No. of assays	No. of segments clotted	Mean serum cholesterol, mg %
Mash	24	9	140
16% corn oil + 0.8% cholesterol	12	5	492
16% coconut oil + 0.8% cholesterol	6	2	781
8% olive–8% coconut + 0.8% cholesterol	6	3	639
Total—Purified diet	24	10	601

Table II shows the results when a single lot of pooled human serum was infused into birds raised on commercial mash as well as on various types of purified diets. The latter produced considerable differences in degree of hypercholesterolemia. None of the differences is significant (Chi Test).

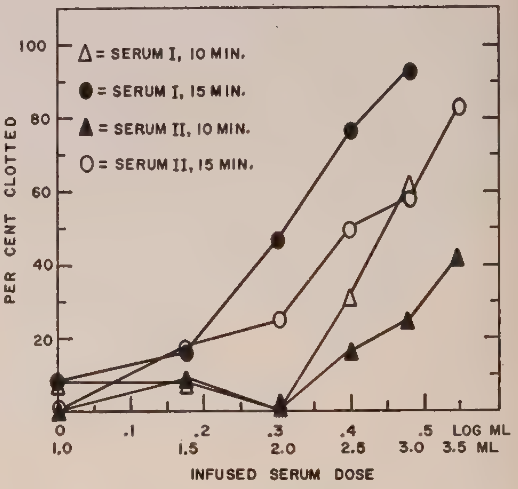

FIG. 1. Assay of 2 lots of pooled human sera with 10 and 15 min. endpoints.

Discussion. That chicken serum is inactive in this system confirms Wessler's finding that Hageman Factor and Plasma Thromboplastic Component are necessary for inducing intravascular thrombosis. Didisheim *et al.* (12) have previously shown that chickens have no detectable Hageman Factor and levels of Plasma Thromboplastic Component equivalent to less than 5% of normal human serum values.

Levels of serum necessary to produce thrombosis are higher in our experiments with chickens (10 ml/kg) than reported by Wessler for rabbits (1.32 ml/kg); duration of segment isolation in the rabbits was 10 minutes. In a small series of experiments on adult rats, we have found that human serum infusions of 1.25 mg/kg will produce thrombosis within 2 minutes. Chicken serum is inactive in the rat.

Whether the difference between the 2 human serum lots was due to real differences between the lots of serum or to variation between the two groups of assay birds is not known. Until the sources of variation are known, this sort of assay should be done on a single group of birds within a short period of time.

Gross alterations in the plasma lipids of assay birds failed to cause any differences in the clotting rates. Until infused sera from patients with hyperlipidemias or recent thrombotic episodes can be assayed, one cannot exclude a role for lipids in this system. The system described is suitable for quantitation and use of this approach may facilitate the evaluation of the role of lipids in intravascular thrombosis.

Summary. (1) A method of producing serum-induced intravascular thrombi has been adapted to use in a convenient laboratory animal, the chicken. (2) Dose-response curves can be constructed and statistically compared. (3) The factors influencing the clotting rates are not all known, but alterations in serum lipids in the assay animals did not influence the results.

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Received Sept. 1, 1960. P.S.E.B.M., 1960, v105.

Survival of Calves Treated with Autologous Bone Marrow After Exposure To Lethal Dose of Whole-Body Irradiation.* (26096)

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In conjunction with earlier experiments on induction of aplastic anemia in calves by trichloroethylene-extracted soybean oil meal (1) or by S-(dichlorovinyl)-L-cysteine(2), we attempted, without success(3), to prevent development of the blood dyscrasia by administration of D,L-batyl alcohol. Inasmuch as the toxic agent, if given in sufficient amounts and without therapeutic treatment, needs to be administered for only a short period of time(2,3) to damage the blood forming organs irreversibly, it appeared possible that hematopoiesis might be restored by intravenous injection of homologous bone marrow or other hematopoietic tissues. Bone marrow transplants have been effective in several species for recovery of hematopoietic function after the bone marrow had been damaged by lethal doses of X-, gamma- or neutron-irradiation(4). In mice homologous fetal liver has been reported(5) to afford greater protection against radiation damage than adult homologous bone marrow. Administration of bone marrow has also been used against the toxic effects of some "radiomimetic" compounds which damage the bone marrow. Thus, treatment with isologous bone marrow afforded protection of rats against 1,4-dimethanesulfonoxybutane(6,7) and of mice against one of its higher homologs(8). Tran Ba Loc *et al.*(9) found that isologous but not homologous bone marrow or spleen protected mice against the nitrogen mustard, methyl-bis-(2-chloroethyl) amine. Kawamoto(10) reported that treatment of mice with isologous bone marrow gave some protection against toxic effects of urethan.

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* Paper No. 4467 Scientific Journal Series, Minn. Agr. Exp. Station. Supported in part by funds from U. S. Atomic Energy Commission and U. S. Public Health Service. The assistance of Dr. M. K. Loken with dosimetry and of C. R. Lagerquist, H. W. Moon and H. A. Pattison with other aspects of this work is gratefully acknowledged. We are indebted to Dr. F. D. Knipling, Princeton, Minn. for keeping the heifers on his farm since April 1960, and for his assistance with subsequent clinical observations.

In our experience(11) with calves in which severe bone marrow damage was induced by feeding of ½ lb trichloroethylene-extracted soybean oil meal per day per 100 lb bodyweight for 10 days(3), the intravenous injection, on the second or fifth day after withdrawal of the toxic feed, of homologous bone marrow in amounts containing 1.1 to 4.9 x 10^{10} nucleated cells failed to alter the course of subsequent fatal blood dyscrasia.

Inasmuch as there is no previous record, to our knowledge, of attempts to transplant bone marrow in bovine animals it was important to determine whether this procedure could afford in this species, as it has in several others, protection against radiation-induced aplastic anemia.

Procedures. Female Holstein calves weighing initially about 50 kg were managed, conditioned and then exposed to a lethal dose of 250 roentgens (tissue exposure dose at the midline) gamma-radiation under exactly the same conditions as those previously described (12). Hematologic examinations were made daily for 3 months, later less often, with blood collected from a jugular vein. Tissues used for therapeutic treatment were collected and injected as follows:

Fetal tissues. The fetuses, with an estimated age of 3½ to 4½ months were obtained at slaughter in a local abbatoir and brought to the laboratory for removal of tissues under aseptic conditions within one hour after death of the cow. Liver, spleen and thymus, with average weights of 31.9, 2.1 and 1.3 g respectively were homogenized gently, for 10 seconds, with a small volume of cold Geys'-citrate solution(13), strained through a nylon gauze and the fluid was injected, intravenously, about 19 hours after completion of the irradiation. The injected suspensions contained from 7.8 x 10^9 to 9.1 x 10^{10} nucleated cells with an average of 2.9 x 10^{10}, about half of which were hemic cells.

Homologous bone marrow. Immediately after slaughter of normal female Holstein calves, bone marrow was squeezed with the aid of a mechanics' vise from the ribs and from segments of the sternebrae and vertebral column. The marrow was collected in cold Geys'-citrate solution and filtered through a sterile nylon gauze to remove fat and gelatinous clots. The filtered suspension was injected intravenously into calves about 19 hours after completing their irradiation. The injected bone marrow contained from 2.1 to 4.7 x 10^{10} nucleated cells.

Autologous bone marrow. This was collected with heparinized syringes by biopsy of 18 to 20 sites on the sternum and ribs, about 2 hours before the animal was irradiated. The collected marrow was diluted with cold Geys'-citrate solution and stored cold until injected, intravenously, within 1 hour after completion of the irradiation.

All injected suspensions contained 500 units of penicillin per ml. They were injected at a rate of about 5 ml per minute. The volumes of the injected suspensions ranged from 50 to 375 ml.

Results. In general the calves tolerated the injections well; only 2 cases of temporary respiratory distress were observed after treatment with homologous bone marrow. As shown in Table I all calves which received homologous bone marrow, fetal tissues or no therapeutic treatment died about 20 days after irradiation, or were slaughtered in moribund condition. There was no evidence that homologous bone marrow or the fetal tissues modified in any way the course of the "bone marrow syndrome" or radiation damage. No indications were obtained that the injected

TABLE I. Effect of Bone Marrow or Fetal Tissues on Survival of Calves Treated with 250 r Whole-Body Gamma Radiation.*

	Therapeutic treatment, I.V.		
No. of calves	Substance	Mean No. nucleated cells	Mean days until death
10‡	None	None	19.4 ± 2.9†
5	Homologous bone marrow	3.8 × 10^{10}	18.4 ± 1.6
5	Mixture of homologous fetal liver, spleen, thymus	2.9 × 10^{10}	20.2 ± 3.1
6	Autologous bone marrow	1.2 × 10^9	23.1 ± 4.8
9	*Idem*	1.5 × 10^9	Survived

* Calculated as tissue exposure dose at the mid-line.

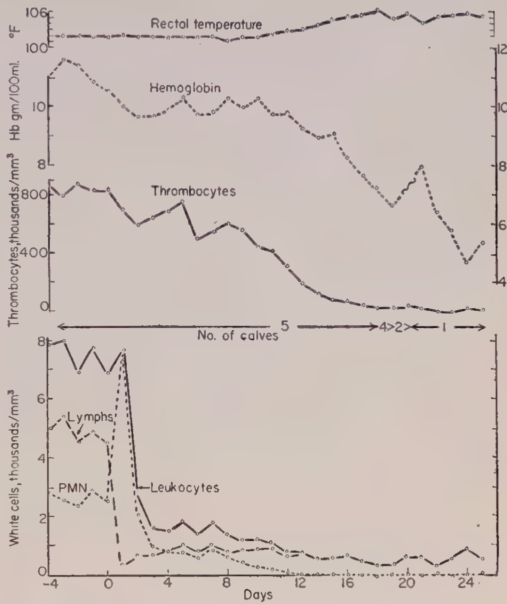
† Stand. dev.

‡ Animals referred to previously(12).

cells were able to proliferate in the irradiated host and repopulate the hematopoietic centers. Period of survival after irradiation was not prolonged by the treatment.

In five of the 15 calves which received autologous bone marrow the blood dyscrasia also took a fatal course. The changes in the hematologic values of these 5 calves are shown in Fig. 1. They coincide essentially with those previously reported(12) for the uninjected calves, and with those observed in the calves treated with homologous bone marrow or fetal tissues.

In contrast to this, 10 calves which were also injected with autologous bone marrow, after a severe hematologic crisis between the 14th and 20th day made a hematologic recovery as shown in Fig. 2. Their rectal temperatures never reached the high values of up to 107° observed in calves which failed to recover. One of these animals started to show rapid hematologic recovery involving all blood elements after the 22nd day but died from rupture of a coronary vessel, with fatal hemorrhage on the 28th day; it is not included in Table I in the group shown to have survived the bone marrow syndrome. In the other animals there was a gradual increase in lymphocytes and the polymorphonuclear leukocytes as shown in Fig. 2. After the 40th day the thrombocyte counts became stabilized at about 450,000 per cmm which is somewhat subnormal for calves of this age. No external evidence of radiation damage



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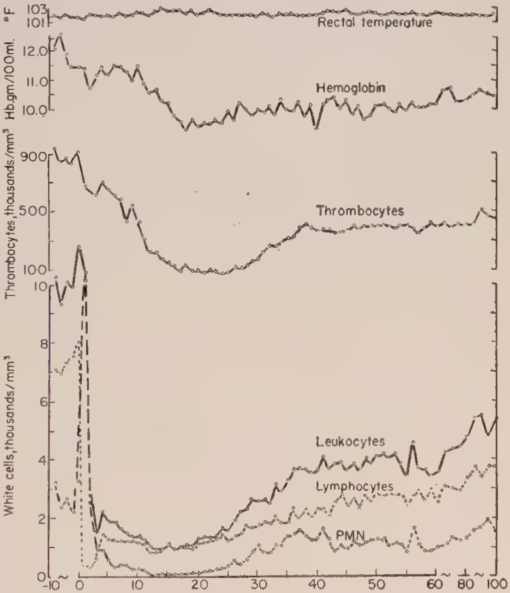

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FIG. 1. Hematologic changes in "non-survivors" following exposure to lethal dose of gamma-radiation and I.V. inj. of autologous bone marrow.

FIG. 2. Hematologic changes in "survivors" following exposure to lethal dose of gamma-radiation and I.V. inj. of autologous bone marrow.

such as clouding of the cornea, depilation or change in hair color was ever observed.

The subsequent development and status of the calves which survived after treatment with a lethal dose of gamma-radiation and autologous bone marrow is of interest. After 60 days they were fed a ration of alfalfa hay supplemented with a grain mixture and kept outdoors most of the time. Since spring of 1960 they have been on pasture. One animal ingested a foreign body and was slaughtered because of ensuing traumatic pericarditis and reticulitis, 11 months after irradiation. The 8 other animals developed normally and had estrous cycles with regular intervals. They have been bred by artificial insemination and seven are now (19 to 27 months after irradiation) pregnant. At various times their blood counts have reached levels which are considered normal for heifers of this age. There has been a tendency toward thrombocytopenia in 4 of the animals with counts of 200,000 to 300,000 per cmm being recorded several times. No evidence of leukemia has been obtained. With the exception of 1 heifer which is somewhat gaunt, the animals appear to be normal.

Summary. Intravenous injection of homologous bone marrow or of a mixture of fetal liver, spleen and thymus after exposure to a lethal dose of whole-body gamma-radiation failed to protect calves against death from bone marrow damage. Nine of 15 calves treated with autologous bone marrow recovered after a severe hematologic crisis; 8 of these developed normally, showed no apparent signs of late radiation damage, had regular estrous cycles and 7 are now pregnant.

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Received Sept. 2, 1960 P.S.E.B.M., 1960, v105.

Trachoma Viruses Isolated in United States. 3. Strain specific effects of tetracycline and penicillin.* (26097)

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Members of the psittacosis—LGV—trachoma group of viruses differ from "true" viruses in being susceptible to the action of many antimicrobial drugs both experimentally and clinically. Long before trachoma viruses were first successfully cultivated(1) millions of persons in trachomatous areas were being treated with topical tetracycline or sulfonamide applied to the eye. Under the auspices of the World Health Organization extensive trials of various time-dose regimens

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* Supported by grants from Nat. Inst. Health, Burroughs, Wellcome Fd. and Res. Comm. of Univ. California.

in trachoma treatment are being conducted (2). In laboratory studies(1,3) it has been observed that virus proliferation was completely inhibited by tetracyclines and penicillin in high concentrations, partially inhibited by chloramphenicol and unaffected by streptomycin. These findings formed the basis for the now uniform usage of streptomycin in isolation of trachoma viruses from clinical specimens(4,5). In view of reports of striking failures of penicillin to eradicate trachoma virus from experimentally induced infection in man(6) we undertook quantitative studies of antimicrobial drug action on trachoma viruses. This report deals with the effect of tetracycline and penicillin on 3 representative strains isolated in United States.

Materials and methods. Viruses: Three representative strains of trachoma virus isolated in United States(5) were employed. To evaluate possible strain-specific behavior, pools from 2 yolk sac passage levels (YS) of each isolate were used with the following egg LD_{50} titers: Strain BOUR, YS 10 ($10^{-6.4}$), YS 12 ($10^{-4.4}$), strain ASGH, YS 7 ($10^{-7.4}$), YS 8 ($10^{-6.7}$), strain APACHE #1, YS 7 ($10^{-6.7}$), YS 8 ($10^{-5.8}$). Stock virus pools were prepared as described(7) and stored at –40°C. Bacterial sterility was established by suitable cultures.

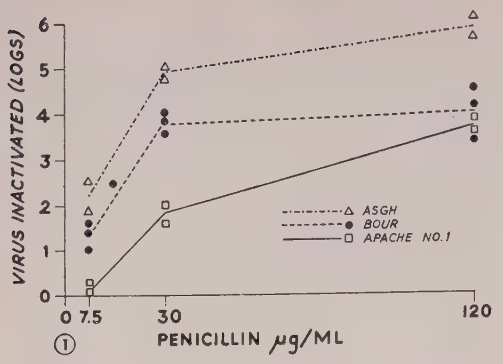
Antibiotics. Crystalline potassium penicillin G was obtained commercially in sterile vials containing 1 million units. Stock solutions of 50,000 units/ml (30 mg/ml) were prepared in saline freshly for each test. Tetracycline hydrochloride in sterile vials containing 20 mg was supplied by Dr. F. Fink. Stock solutions of 1 mg/ml were prepared in saline freshly for each test. Further dilutions of antibiotics were made in broth and kept refrigerated until mixed with virus dilutions. Drug concentration is expressed as μg/ml in the final drug-mixture that was inoculated into eggs. No additional doses of drug were administered.

Assay of antibiotic activity. A vial of stock virus was rapidly thawed, 10-fold dilutions were prepared in chilled broth and promptly added to the antibiotic dilutions. The mixture was kept for 30 minutes in an ice bath, then inoculated in 0.5 ml volume into the yolk sac of groups of 7-day embryonated eggs. Five or 6 eggs were used per drug-virus mixture. Eggs were incubated at 35°C and candled twice daily. Deaths occurring during the first 48 hours were discarded as non-specific. All deaths from the third to the twelfth days of incubation were recorded. Random yolk sacs from dead eggs in each group were smeared and stained by Macchiavello's method to confirm microscopically presence of virus. Such virus was seen regularly in eggs dying on or after the 6th day of incubation. Deaths between day 3 and 5 of incubation were exceedingly rare, and in them virus was seen infrequently. Fifty per cent death endpoints were calculated by accepted methods(8) and expressed as egg lethal titer (ELD50)/g of yolk sac. Each test included a series of virus dilutions mixed with antibiotic-free broth. Final results are presented as the amount of "virus inactivated" by a given concentration of antibiotic (*i.e.*, difference between ELD50 of antibiotic-free and antibiotic-containing virus mixture).

Results. For simplicity of presentation amounts of "virus inactivated" (logs) were plotted against concentration of drug (arithmetic scale). This presentation seemed preferable as it yielded a number of linear or parallel relationships whereas no simple relationships were apparent on log-log plots. At least 2 different stock virus pools with different egg LD_{50} titers were titrated in the presence of varying amounts of each drug. In this fashion it was hoped that the behavior of a given single virus pool might not be mistaken for a strain characteristic.

The results with penicillin are shown in Fig. 1, those with tetracycline in Fig. 2. For each strain the results appeared remarkably constant, as evidenced by the proximity of individual points in the figures. Each strain appeared to behave in an individual and reproducible manner.

Penicillin (Fig. 1): At each of 3 concentrations strain APACHE #1 was markedly more resistant than strain ASGH, by 2-3 logs, with strain BOUR in an intermediate position. Thus penicillin 7.5 μg/ml had no effect on APACHE #1, but "inactivated" 99%



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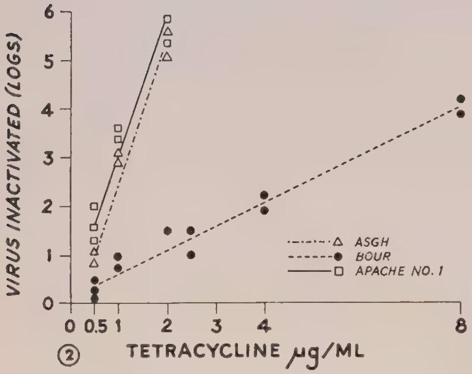

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FIG. 1. Effect of penicillin on 3 strains of trachoma virus. Amount of virus ''inactivated'' (Log ELD_{50} without antibiotic − log ELD_{50} in presence of antibiotic) by various concentrations of penicillin G potassium.

FIG. 2. Effect of tetracycline on 3 strains of trachoma virus. Amount of virus ''inactivated'' (log ELD_{50} without antibiotic − log ELD_{50} in presence of antibiotic) by various concentrations of tetracycline hydrochloride.

of ASGH. The 4-fold increase in penicillin concentration from 7.5 to 30 µg/ml reduced viable virus of any strain by 99%, but the next 4-fold step had much less effect. At a penicillin concentration of 800 µg/ml (not shown in chart) no virus-specific deaths were observed. Thus 800 µg/ml penicillin completely blocked viral proliferation to lethal levels, in spite of drug inactivation during incubation at 35°C. No blind passage of surviving eggs was done and thus the persistence of sublethal amounts of infective trachoma virus cannot be ruled out. However, smears of surviving eggs showed no microscopic evidence of virus. Fig. 1 suggests that different strains of trachoma virus have basically different susceptibility to penicillin and that in each strain members of the population vary in penicillin-susceptibility over a fairly wide (more than 16 fold) range.

Tetracycline (Fig. 2): The response of the 3 strains of trachoma virus to tetracycline appears to differ markedly from their response to penicillin. For any one strain the relationship of drug dose (arithmetic) to amount of "virus inactivated" (logs) appears to be linear, and the slopes of the lines are distinct for a strain. Whereas 0.5 µg/ml tetracycline affects strains BOUR and ASGH only to a negligible extent, it reduces the infective titer of APACHE #1 by from 20 to 99%. A concentration of 2 µg/ml tetracycline completely prevents growth of strains ASGH and APACHE #1 but affects BOUR little. Even 8 µg/ml does not completely "inactivate" BOUR. Thus it appears that 2 strains are completely "inactivated" over a very narrow (4-fold) range of tetracycline, apparently all infective units being uniformly susceptible to that drug. Strain BOUR, on the other hand, is "inactivated" over a very wide range of drug concentrations, suggesting that some infective virus units are markedly more resistant to tetracycline than others.

Discussion. The results reported here do not at present have any direct practical applicability to treatment of human trachoma. Ophthalmic ointments or solutions usually contain tetracycline 10 mg/g. Thus the concentration of drug placed on trachoma-infected epithelial cells is many times higher than the concentration necessary for *in ovo* inhibition of growth of the more resistant virus strains. However, rapid removal of topically applied preparations from the conjunctival surfaces by lid action and uncertainty of diffusion and penetration might result in barely effective concentrations of tetracycline within infected epithelial cells. Under such circumstances differences in strain susceptibility might make themselves felt in the clinical response of the patient. A much more important aspect of these strain differences might pertain to the suppression versus eradication of trachoma infection in the eye. Intracellular drug concentrations might be sufficient to interfere completely with the multiplication of the more susceptible strains, yet might permit the survival and slow proliferation of the more resistant strains. While it has been reported that penicillin is effective in trachoma(9), controlled experimental evidence(6) suggests that it often fails to eradicate the infection. This result might be anticipated from our laboratory observations that even 120 μg/ml penicillin failed to completely destroy infectivity of some strains while as little as 7.5 μg/ml reduced infectivity of other strains by 99% or more. The shape of the lines in Fig. 1 suggests that the curves might become asymptotic when complete block of virus proliferation is approached at high drug levels. Thus it might be expected that very high concentrations of penicillin would largely but not completely suppress virus multiplication. Expressed clinically this would mean amelioration of the disease without cure, and a high probability toward relapse—as was indeed observed in patients(6). It may be remembered in this connection that T'ang(1) isolated some trachoma viruses from clinical specimens containing 30-120 μg/ml penicillin, perhaps because in conjunctival scrapings rich in trachoma virus some particles might retain infectivity even after exposure to penicillin.

Apart from possible practical implications, the demonstration of marked strain-specific effects of tetracycline and penicillin has several points of interest. The selection of very highly drug-resistant mutants may be possible and their use as "markers" for genetic studies suggests itself(10). Antibiotic action should be related to growth cycles of trachoma virus in eggs to compare the inhibition dynamics of these large viruses to those observed with bacteria. For example it is possible that the high susceptibility of strain ASGH to penicillin is a function of its rapid growth rate, as an analogy of the dependence of penicillin effect upon growth rate of bacteria. Evidence has been presented(7) that ASGH indeed kills eggs faster than other strains of trachoma virus, perhaps because of faster growth rate.

All virus inocula employed in the present work contained streptomycin. The effects described may therefore represent combined antibiotic action rather than the effect of the single drug under study. While 20 mg/ml streptomycin has no demonstrable effect on trachoma virus infectivity *in ovo*(3,4,5) and while Gordon *et al.*(10) failed to demonstrate any combined antibiotic effect on psittacosis viruses, the possibility exists that biologically inactive drug may enter into combined action with another drug(11). Therefore future drug assays must include truly antibiotic-free preparations of trachoma viruses.

Summary. The ability of varying amounts of penicillin or tetracycline to interfere with the growth of trachoma virus in eggs was studied. Three strains of this agent isolated in the United States behaved in a reproducible and individual manner toward these drugs. They exhibited a very large range of differences in susceptibility to these antibiotics. The theoretical and practical implications of these findings are discussed.

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Received Sept. 6, 1960. P.S.E.B.M., 1960, v105.

Chemical Dissection of Rat Skin.* (26098)

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The primary anatomical components of connective tissue are fibers, cells and ground substance(1). Hydroxyproline is a quantitative measure of the major fiber, collagen (2), while hexosamine is a measure of ground substance mucopolysaccharides and glycoproteins(3). Uronic acid is largely confined to the acid mucopolysaccharides(4). Proteins and, to a lesser extent, urea, nitrogenous bases, hexosamine and amino acids contribute to total dermal nitrogen content.

This paper describes both the analysis of cleaned, shaven rat skin with respect to these materials and water and fat, and the extractive dissection of rat skin into ground substance, neutral soluble collagen(5), procollagen(6) and insoluble collagen.

Material and methods. Twelve male Sprague-Dawley rats averaging 330 g in weight were sacrificed by etherization and exsanguination, and after shaving about 5.5 g of abdominal skin were removed and dissected clean of adhering fat, fascia and muscle. After mincing, the tissue was either lyophilized or stored in the frozen state. Subsequently, portions of these tissues were hydrolysed at 100°C for 8 hr in 10 ml/g of 4N HCl. The acid hydrolysates were then analysed for hexosamine(7) uronic acid(8) and hydroxyproline(9). Nitrogen was determined by digesting in triplicate 0.10 ml of a 1:10 dilution of acid hydrolysate with 0.2 ml of 50% H_2SO_4 containing 1% SeO_2 at 270°C in a Halikanen digestion box for 2 hours, and Nesslerization(10). These conditions for hydrolysis and digestion were optimal, and quantitative recoveries of added collagen, mucopolysaccharide, hydroxyproline or hexosamine could be effected thereby(11).

The remainder of the tissue samples were extracted for 45 minutes at 4°C with 10 ml/g of 0.15 M NaCl at 17,500 rpm in a Lourdes Tissue Blender and the homogenate allowed to stand over night in the cold. After centrifugation at 25,000 × g for 1 hr. the clear supernatant was removed, and the residue reextracted similarly with 0.50 M NaCl. The residue from this second extraction was in turn extracted with 20 ml/g of 0.50 M citrate buffer (pH 3.6) under similar conditions.

Repeated extraction of rat skin with each solvent resulted in the further release of less than 15% of the hydroxyproline or hexosamine found in the first extract under the conditions described above.

The hydroxyproline, hexosamine, nitrogen and uronic acid content of these extracts was determined after HCl hydrolysis as described above.

Results and discussion. Theoretically, most of the free mucopolysaccharides and glycoproteins of the ground substance, and contaminating serum glycoproteins, would be soluble in isotonic saline. The residue of this extraction contained morphologically intact cells and collagen, but no longer stained profoundly with Alcian blue, a histochemical reagent for acid mucopolysaccharides(12). The chemistry of this extract of rat skin has been exhaustively studied by Sweeney(13) and Mathieson(14).

The extrafibrillar precursor of collagen is soluble in 0.5 M NaCl(5). The residue from this extraction contained collagen fibers which, under the electron microscope, had lost a portion of their surface material and appeared somewhat "leached." The cells had been distorted and destroyed.

Citrate soluble procollagen from rat skin has been studied in detail by Orekovitch and Shpikiter(6). The residue after extraction by this solvent contained grossly swollen, distorted collagen bundles and gluey masses of fibers. Subtraction of the sum of the various soluble components from the appropriate total concentration found in the whole skin permits determination of the insoluble dermal constituents to be made. The residue of the sequential extraction procedure described

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* Supported by N.I.H. grant.

TABLE I. Extractive Dissection of Normal Rat Skin.

Extract	Hydroxyproline, μmoles/g	Hexosamine, μmoles/g	Nitrogen, mmoles/g
.15M NaCl	7.7 ± 3	5.1 ±.3	1.30 ±.06
.50M ”	29 ± 2	2.4 ±.1	1.20 ±.06
.50M citrate buffer	12 ± .6	.10	.60 ±.04
Insoluble	312 ±13	4.4 ±.2	3.90 ±.2
Total	360 ±15	12.0 ±.6	7.00 ±.3

above was so swollen (9×) and non-homogeneous that direct measurements were singularly unreliable. The results are presented in Table I.

Because of the large amount of color contributed to the carbazole method by dermal hexoses, uronic acid determinations were unreliable. Quantitatively, however, most of the uronic acid was found in the isotonic saline extract, and very little in the other soluble fractions. Table I then is concerned solely with the hexosamine, hydroxyproline and nitrogen content of the various fractions. More than 42% of the total dermal hexosamine was soluble in isotonic saline, while only a trace of this amino sugar appeared in the citrate fraction. About 37% of the hexosamine was completely insoluble and presumably represents either a glycoprotein or a mucopolysaccharide bound to the collagen fibers.

Purified collagen contains about 18.6% nitrogen or 13.3 μmoles/mg and 13.6% hydroxyproline or approximately 1.1 μmole/mg (15). Ratio of hydroxyproline to nitrogen (μmole/mmole) for highly purified collagen is about 83. A similar ratio for the insoluble dermal material is about 80, therefore this component is almost pure collagen. The extra nitrogen contaminating this fraction probably results from keratin and elastin. The purity of this collagen precludes the insoluble hexosamine being glycoprotein, since this material would contain almost as much nitrogen as simple protein. Therefore insoluble collagen is intimately associated with mucopolysaccharide. This is consistent with the claim of Stoianova *et al.*(16) that collagen fibrils are surrounded by a sheath of mucopolysaccharide which is removed by hyaluronidase activity.

The non-protein nitrogen of normal skin was studied by measuring nitrogen content of the trichloracetic acid supernatant of the saline fractions. Five percent of the nitrogen in the isotonic saline extract and 3% of the 0.5 M saline soluble nitrogen was not precipitated by this treatment.

The amount of dermal nitrogen accounted for by collagen may be determined by multiplying the hydroxyproline content of the tissue, since 1 μmole equals 1 mg of collagen, by 13.3. Dermal collagen accounts for about 70% of total nitrogen. The difference between total nitrogen and collagen nitrogen would be due largely to non-collagenous protein and glycoprotein.

By lyophilization, it was found that fresh, cleaned, shaven skin contained about 550 mg/g H_2O, and after standing in the frozen state for 5 days, all skin samples contained 250 mg/g. Further storage for 25 more days did not appreciably decrease this value(11). The data of Table I was obtained using stored skin, and could be corrected with respect to water loss *via* sublimation during storage in the frozen state by multiplication by 0.68(11). These corrected values agreed within 10% with those obtained using fresh tissue. Accordingly, fresh rat skin contained 240 mg of collagen, 150 mg of other protein and 550 mg of water per g. The difference between 1000 and the sum of these 3 dermal constituents must be accounted for by fat, or 60 mg/g. Kao *et al.*(17) have reported total fat content of the skin of 8-month-old male rats (approx. 230 g) to be 74 mg/g.

Summary and conclusions. Extraction of rat skin with 0.15 M NaCl in the cold removes almost half of the dermal hexosamine and most of the uronic acid, leaving a residue which no longer stains profoundly with Alcian blue, although cells and collagen of the tissue are intact. Further extraction with 0.50 M NaCl removes about 8% of total dermal hydroxyproline leaving a residue in which neither the cells nor the collagen are intact. The citrate buffer soluble hydroxyproline (3% of the total) is not contaminated with hexosamine, while ⅓ of total dermal amino sugar is found intimately associated with the insoluble residue, probably as a mucopolysaccharide. This residue is highly purified dermal collagen. Finally, fresh skin contains 55% water, 6% fat, 24% collagen and 15% non-collagen protein and glycoprotein.

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Received Sept. 6, 1960. P.S.E.B.M., 1960, v105.

Metabolism of Cortisol by the Liver of Sodium Deficient Rats.[‡] (26099)

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A major step in the metabolism of corticosteroids is reduction of the Δ^4-3-ketone group(1). It has been shown that rat liver contains steroid Ring A hydrogenases capable of catalyzing this reduction(2). Urquhart *et al.*(3) and Yates *et al.*(4) have proposed that rate of hepatic reduction of adrenal cortical steroids is a controlling factor in rate of secretion of those steroids by the adrenal through a negative-feedback mechanism by which the anterior pituitary (ACTH) regulates plasma corticosteroid concentration. It is likely then that conditions which produce changes in adrenocortical secretion rates might also alter the ability of the liver to metabolize active corticosteroids.

Eisenstein and Hartroft(5) have shown that total steroid secretion by the isolated adrenal of sodium deficient rats was significantly less than that of controls. The purpose of this study was to evaluate the effect of sodium deprivation on capacity of rat liver to reduce the Δ^4-3-ketone group of cortisol.*

Methods and materials. Female white rats of the Carworth strain (maintained on Purina pellet chow), weighing 140-200 g, were divided into two groups. One group (21 rats) was fed a commercially† prepared synthetic low-sodium diet which included all the nutrients essential for normal growth except sodium and contained 14.6 mg Na^+/100 g of diet and 910 mg K^+/100 g of diet. The other group (20 rats) was fed a diet† similar in all respects to the low-sodium diet except that

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[‡] This study supported in part by grants from U.S.P.H.S.

* Δ^4-pregnene-11β,17α,21-triol-3,20-dione.

† Nutritional Biochemicals Corp.

TABLE I. Effect of Sodium Deprivation on Rat Liver Steroid Hydrogenase Activity.

Group*	No. rats	Days on diet, avg	μg/cortisol reduced/g liver/min.
Control	20	35	40.7 ± 7.4†
Low Na	21	60	30.6 ± 6.3
			$P < .01$

* Avg intake of Na for control group 1.60 meq/day; low-sodium group 0.16 meq/day.

† Stand. dev.

it included normal amounts of electrolytes. It contained 148 mg Na$^+$/100 g of diet and 500 mg K$^+$/100 g of diet by analysis and will be referred to as the normal synthetic diet. Information pertaining to length of the regimen and average sodium intake is indicated in Table I. The animals were killed by decapitation, and liver, blood and muscle samples obtained. The liver was rinsed in iced saline solution, blotted and weighed. It was then homogenized in a solution of 0.25 M sucrose with 0.04 M nicotinamide (4 ml/g liver). The liver homogenate was centrifuged at 7000 × g for 15 minutes and the supernatant fraction was used for assay of the steroid hydrogenase. Two ml of this supernatant fraction were added to 3.5 ml of a solution(6) containing the necessary co-factors for TPNH generation. One tenth ml of a 0.01 M solution of hydrocortisone in ethanol was used as the substrate and added to each flask. The samples were incubated in a Dubnoff shaker for 15 minutes at 38°C in an atmosphere of nitrogen and then 20 ml of chloroform were added. To measure the difference in enzymatic reduction of the hydrocortisone, comparable control tubes were set up in which the substrate was added after addition of 20 ml of chloroform. The samples were extracted by shaking for 10 minutes and the chloroform layer was washed with 0.1 N NaOH and 0.1 N HCl. Aqueous layers were removed by aspiration. Five ml aliquots were taken to dryness under reduced pressure and the residue dissolved in 10 ml of methanol.

The ultraviolet absorption was determined in a Beckman DU spectrophotometer at 242 mμ. The difference between the optical density of the controls and the samples was used to calculate the amount of Ring A reduction which had taken place.

Results. The results presented in Table I show that the capacity of the livers of sodium deficient rats to reduce Ring A of cortisol is greatly reduced when compared to animals maintained on a normal synthetic diet. The livers of sodium deficient animals had about 25% less steroid hydrogenase activity than control animals. This difference was found to be highly significant ($P<0.01$). Table II shows electrolyte content of plasma and muscle of both groups. The sodium deficient group had significantly less sodium and significantly more potassium in their skeletal muscle than the control group. There were no significant differences in plasma sodium and potassium levels, which is to be expected since plasma sodium and potassium levels tend to remain within normal limits except in extremely severe chronic sodium deficiency. Muscle electrolyte changes are therefore a better index of sodium deficiency.

Discussion. The deceased capacity of the liver of sodium deficient rats to reduce the Δ^4-3-ketone group of cortisol could have been the result of: (a) a decreased requirement for Δ^4-hydrogenases due to a diminished steroid output from the adrenal; or (b) a reduced enzyme concentration or activity within the liver cells elicited by the stress of salt deprivation. If the latter possibility is correct, a decreased secretion of adrenal steroids by salt deprived rats(5) can be explained, at least in part, by the feedback stabilization of plasma corticosteroids by the hepatic-hypo-

TABLE II. Electrolyte Content of Skeletal Muscle and Plasma.

	Skeletal muscle, meq/100 g (wet wt)		Plasma, meq/l	
Group	Na	K	Na	K
Control	$3.80 \pm .71$*(12)†	$9.58 \pm .73$ (12)	142 ± 5 (20)	$7.0 \pm .7$ (20)
Low sodium	$2.92 \pm .37$ (19)	$10.3 \pm .5$ (19)	145 ± 4 (19)	7.5 ± 1.1 (12)
	$P < .01$	$P < .01$		

* Stand. dev.

† No. in parentheses represents No. of values obtained.

thalamic-pituitary-adrenal axis described by Urquhart *et al.*(3) and Yates *et al.*(4). It is also possible that both mechanisms occur simultaneously. However, the results of this study do confirm previous evidence that the liver plays an important role in regulation of plasma corticosteroid levels.

It is not likely that the observed decrease in hepatic metabolism of cortisol by these animals would precipitate an adrenal crisis during stress because there still remains a considerable capacity for steroid reduction in the salt deficient rats(5). Further studies under more severe conditions of sodium restriction and for longer periods might be valuable in assessing this effect in chronic sodium deficiency.

Summary. A decreased capacity of liver homogenate to metabolize cortisol in sodium deficient rats has been demonstrated. The rate of Ring A reduction was diminished by about 25% below that of control animals. The possibility that this effect may result in a decreased adrenal corticosteroid secretion *via* the negative-feedback mechanism by which plasma corticoid levels are stabilized is discussed.

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Received Sept. 9, 1960. P.S.E.B.M., 1960, v105.

Vi Antigens of the Enterobacteriaceae. IV. Purification and Properties of Vi Antigen of *S. paratyphi C*.* (26100)

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Isolation and purification of relatively undegraded Vi antigen by mild methods from *Salmonella typhosa, Escherichia coli* and *Paracolobactrum ballerup* has been reported by Baker *et al.*(1). Production of Vi antigen by some strains of *Salmonella paratyphi C* was reported by Rouchdi(2). However, neither the technic of purification mentioned above(1) nor that devised by Webster *et al.* (3,4) proved useable for purification of the Vi antigen of *S. paratyphi C*. In the present communication a method for the purification of the Vi antigen of this organism is outlined together with chemical and biological properties of this antigen.

Methods. The culture of *S. paratyphi C* (C-32) used was obtained from Dr. F. Kauffmann. The organisms were grown, as previously described(1), on a casamino acid medium containing only dialysable components. After about 18 hours growth the cultures were killed by addition of 1.5 to 2 volumes of acetone (technical grade), dehydrated by washing with acetone, and dried in air.

Nitrogen was determined by the usual micro-kjeldahl method(5), total acetyl as described by Kabat and Mayer(5), O acetyl by a modification of the method of Pippin, McCready and Owens(6), rhamnose by the method of Dische and Shettles(7), hexose by the method of Dische, Shettles and Osnos (8), and phosphorus by the method of Allen (9). Viscosities were determined by means of an Oswald pipette. The Ouchterlony technic was used for the serologic tests which

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* This investigation was carried out under the sponsorship of Commission on Immunization of Armed Forces Epidemiological Board and was supported by Office of the Surgeon General, Dept. of the Army.

TABLE I. Analytical Properties of *S. paratyphi C* Vi Antigen.

Analysis	Lot E + F	Lot G
	%	
Nitrogen	5.85	5.8
Total acetyl	20.6	23.0
O acetyl	12.5	12.5
N " *	8.1	10.5
Rhamnose	.02	.02
Hexose	None	None
Phosphorus	"	"
Relative viscosity†	2.84	2 86

* N acetyl = Total acetyl – O acetyl.
† 0.2% solution in 0.15 M NaCl at 30°C.

were conducted as previously described(10).

Results. Attempts to purify the Vi antigen of *S. paratyphi C* by the previously described method(1) failed. Several preparations, when tested serologically by the Ouchterlony technic, showed the presence of several antigens other than Vi. Preliminary experiments indicated that the contaminating antigens could be eliminated early in the purification by treatment of the crude antigenic extracts with trichloracetic acid. To illustrate the method finally evolved, the following typical experiment is presented.

Seventy-five g of acetone dried bacilli were extracted twice with 750 ml of 0.15 M NaCl at room temperature. To the combined extracts cooled to 5°C, 0.25 N trichloracetic acid was added until no more precipitate formed. The precipitate was removed by centrifugation in the cold and discarded. The supernatant was neutralized to approximately pH 7 by addition of NaH CO_3. The antigens were then precipitated by addition of 2 volumes of ethanol. The precipitate was redissolved in 400 ml of 0.15 M NaCl. At this stage, the material contained at least 4 antigens when tested by the Ouchterlony technic. Further purification was carried out by the method used to prepare other Vi antigens (1). Briefly the method was as follows: A 2% solution of cetyltrimethylammonium bromide was added to the crude material until all of the Vi antigen was precipitated. The precipitate was washed several times with water, suspended in 0.15 M NaCl, and dissolved by addition of alcohol. The Vi antigen was recovered by addition of ethanol until no further precipitate formed (usually 3 to 4 volumes of ethanol were required). The precipitated Vi antigen was extracted at 37°C from the precipitate with 60% ethanol (60% by volume in 0.15 M NaCl). The extraction was repeated 4 to 5 times. The antigen was precipitated from the combined 60% ethanol extracts by cooling to 5°C. The precipitate was collected by centrifugation in the cold, and electrodialysed to remove the last traces of cetyltrimethylammonium bromide. The electodialysed solution was neutralized by addition of NaH CO_3, since the sodium salt was desired, dialyzed against distilled water, and dried from the frozen state. From 75 g of bacilli, 0.4 g of Vi antigen were obtained, a yield of about 0.5%.

Material in the supernatant from the cetyltrimethylammonium bromide precipitate was recovered by addition of 2 volumes of ethanol. This material was free of Vi antigen present in the original extract. This material has not been studied further.

The analytical values of 2 lots of Vi antigen, presented in Table I, were essentially the same as those previously reported for Vi antigens iseolated from *S. typhosa, E. coli* and *P. ballerup*.

The Vi antigen prepared from *S. paratyphi C* was non-toxic for rabbits in doses up to and including 10 mg. It did not prepare

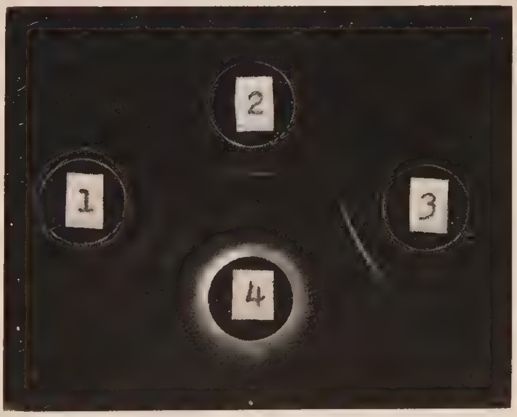

FIG. 1. Comparison of crude and purified *S. paratyphi C* Vi antigens with *S. typhosa* Vi antigen. Reservoir 1 = Crude extract of *S. paratyhpi C*. Reservoir 2 = Purified paratyphi C Vi antigen. Reservoir 3 = Purified *S. typhosa* Vi antigen. Reservoir 4 = *S. paratyphi C* antiserum.

rabbit skin for the Schwartzman reaction in doses of 250 μg. Pyrogenicity of the material was not tested. Serologic tests using the Ouchterlony technic are presented in Fig. 1.

Whereas there were several antigenic components in the crude material, only a single antigenic component was present in the purified antigen tested. Further, it was evident that the Vi antigen from *S. paratyphi C* was identical with that isolated from *S. typhosa.* Similar tests not presented here revealed an identity of the paratyphi C Vi antigen with those isolated from *E. coli* and *P. ballerup.*

Summary and conclusions. A method has been outlined for isolation and purification of the Vi antigen produced by *S. paratyphi C.* Consideration of both the chemical biological properties of this antigen indicated that it is very similar to, if not identical with, those produced by other *Enterobacteriaceae.*

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Received Sept. 15, 1960. P.S.E.B.M., 1960, v105.

Elimination of Circulating Serum Protein Antigens as a Sensitive Measure of Antibody.* (26101)

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The production of antibody following injection of soluble serum protein antigens into animals can be readily detected by the rapid elimination of the antigen from the circulation of the sensitized animal(1,2). This rapid elimination presumably results from the reaction of the circulating antigen with the newly synthesized antibody and uptake of the complex by phagocytic cells(1). Rate of elimination depends on amount of antigen injected, rate of antibody formation and perhaps quality of antibody produced(3). The accelerated elimination of serum protein antigens from the circulation also takes place following both injection of antigen in actively (4,5) and passively(4,6,7) immunized animals and injection of insoluble(8,9) and certain soluble(8) antigen-antibody complexes into normal animals. The purpose of the present investigation was to determine the minimum amount of passively transferred antibody which could be detected by the rapid elimination of I^{131} labeled antigen. The possibility of utilizing the rapid elimination of soluble antigens as a method to measure small amounts of antibody is discussed.

Methods and materials. *I^{131} labeled antigens.* Bovine serum albumin (BSA), Armour and Co., Lot No. S 68108, and bovine serum gamma globulin (BGG), Armour and Co., Lot No. C 904, were labeled with I^{131} (I*) as previously described(10).

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* Publication No. 260 of Department of Pathology, Univ. of Pittsburgh School of Medicine, Pittsburgh, Pa.

Supported by Atomic Energy Comm. contract AT (30-1)-1205.

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Antisera. Rabbit antisera against BSA and BGG were obtained from hyperimmunized rabbits prepared by a series of subcutaneous and intravenous injections of either BSA or BGG, totaling 250-300 mg, given over a 2-4 month period(11). The last injection was given 7 days before bleeding.

Antibody levels of the antisera were determined by the quantitative immunochemical procedures developed by Heidelberger and co-workers(11). Total N in antigen-antibody precipitates and in the various protein solutions was measured by the Markham modification of the micro Kjeldahl technic (12). The pooled anti BSA serum contained 2.18 mg of antibody N/ml, and the pooled anti BGG serum contained 1.20 mg antibody N/ml.

Elimination of I antigen from rabbits.* Albino rabbits weighing 2.5-3 kg were passively immunized by intravenous injection of 1.1-45.0 μg rabbit anti BSA N or rabbit anti BGG N and 24 hours later injected intravenously with 0.1-4 μg I* BSA N or I* BGG N. Control rabbits received comparable amounts of I* antigen but no antisera. Also, non-immunized rabbits received 1-10 mg unlabeled antigen immediately prior to administration of I* antigen in order to determine if a portion of the I* antigens was eliminated by natural globulin capable of acting as antibody. If a small amount of the antigen could be eliminated by natural globulin, the blood of rabbits receiving the prior injection of unlabeled antigen should have contained more I* antigen than the rabbits not receiving a prior injection of unlabeled antigen. Rabbits were bled from ear veins at intervals, and radioactivity of the trichloroacetic acid precipitable fraction of the serum was determined in a NaI crystal scintillation counter and recorded as per cent of total injected protein bound radioactivity remaining in the total blood volume. Sufficient counts were obtained to insure a maximum counting error of no greater than 5%.

One group of rabbits was injected intravenously with a mixture of I* BSA-anti BSA complexes prepared at equivalence with 1 μg antibody N and .18 μg antigen N and incubated at 37°C for 30 minutes prior to injection. Circulating I* BSA-anti BSA complexes were determined as previously described(13), a modification of the method of Farr(14). One ml of 0.15 M NaCl was added to 1.0 ml of serum and mixed, and 2 ml of saturated ammonium sulfate (pH 7.2-7.5) were added to the mixture. After 30 minutes at 0-1°C, the mixture was centrifuged and the supernatant decanted. After 2 washings with 50% saturated ammonium sulfate the precipitate was counted in a NaI crystal scintillation counter. *Elimination of I* antigen-antibody complexes from guinea pigs.* Guinea pigs weighing approximately 500 g were injected intravenously in the lateral penile veins with a mixture of either I* BSA-rabbit anti BSA or I* BGG-rabbit anti BGG prepared *in vitro* in 5% Dextrose in water. Complexes containing .03 to 3 μg antibody N/ml were prepared using weight ratios of 1 μg of BSA N to 5.5 μg of anti BSA N and 1 μg of BGG N to 3 μg of anti BGG N and injected in a total volume of 1 ml. Control animals were injected with comparable amounts of I* antigen but no antiserum. Radioactivity of each animal was determined by counting the whole animal in a large well-type geiger counter immediately after injection and at various intervals. *Elimination of I* antigen-antibody complexes from mice.* Albino mice weighing 20-25 g were treated the same as guinea pigs except that the I* antigen-antibody complexes and I* antigen were injected into the dorsal tail veins. Total volume injected was 1 ml.

All animals used in this study received KI in their drinking water during, and for several days prior to, the experiment.

Results. Elimination of I antigen in sensitized and normal rabbits.* Elimination of I* BSA and I* BGG from both normal rabbits and rabbits passively sensitized with 1.1-45.0 μg of rabbit antibody N is shown in Table I. In the rabbits injected with antibody, the antigen was almost completely eliminated from the blood in 24 hr, while in the control rabbits, 22-34% of the antigen still remained at this time. Ten minutes after injection of I* antigen into rabbits sensitized with 1.1 and 4.5 μg of antibody N, the I* antigen re-

TABLE I. Elimination of I* Antigen from Normal and Passively Immunized Rabbits.†

Antibody N inj., μg	Antigen N inj., μg	% of total inj. I* antigen remaining in blood				% of total inj. I* antigen bound to globulin			
		10 min.	1 hr	6 hr	24 hr	10 min.	1 hr	6 hr	24 hr
45 anti BSA	4 I* BSA	47	13	2	0	46	11	1	0
None	*Idem*	86	67	44	25	3	2	1	0
"	1,500 BSA prior to 4 I* BSA	89	72	44	24	3	2	1	0
45 anti BGG	4 I* BGG	13	10	1	0				
None	*Idem*	93	82	51	28				
"	1,500 BGG prior to 4 I* BGG	87	78	49	26				
22.5 anti BSA	2 I* BSA	36	8		1	37	6		
None	*Idem*	98	79		28	1	1		
"	750 BSA prior to 2 I* BSA	88	75		30	1	1		
22.5 anti BGG	2 I* BGG	33	6		0				
None	*Idem*	89	72		32				
"	750 BGG prior to 2 I* BGG	85	68		29				
4.5 anti BSA	.4 I* BSA	88	43		1	90	42		
None	*Idem*	98	78		30	1	1		
"	150 BSA prior to .4 I* BSA	98	76		26	2	1		
4.5 anti BGG	.4 I* BGG	73	22		2				
None	*Idem*	94	75		30				
"	150 BGG prior to .4 I* BGG	75	64		26				
1.1 anti BSA	.1 I* BSA	93			1				
None	*Idem*	97			27				

† Recorded as avg of results from 2 rabbits.

maining in the blood was considerably higher than in rabbits sensitized with the larger amounts of antibody. In the groups sensitized with 4.5, 22.5 and 45 μg of anti BSA N, almost all of the I* BSA remaining in the blood at the 10 minute bleeding was present in the form of antigen-antibody complexes.

To determine if a portion of the I* antigens was eliminated by natural globulin capable of acting as antibody, 10 mg of unlabeled homologous antigen was injected in some of the normal rabbits prior to injection of the I* antigen. If a small amount of globulin in the serum of immunized animals was capable of acting as a natural antibody, the blood of rabbits receiving the prior injection of unlabeled antigen should contain more I* antigen than the blood of rabbits not receiving a prior injection of excess unlabeled antigen. However, there was no significant difference in level of I* antigen during a 24 hour period in the blood of rabbits receiving the prior injection of antigen and in those not receiving prior antigen (Table I), nor was a significant difference in amount of antigen-antibody complexes detected between the sera of the two groups.

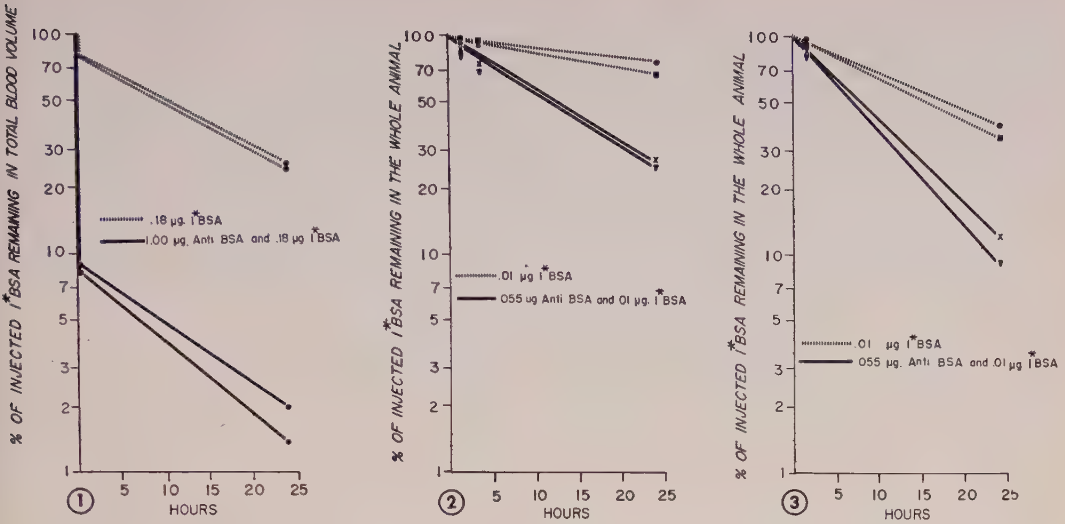
The rapid elimination of I* antigen-antibody complexes prepared at equivalence with 1 μg of anti BSA N is shown in Table II and Fig. 1. As evidenced by the level of I* in the sera, these complexes were almost com-

TABLE II. Elimination of Preformed I* BSA-anti BSA Complexes from Blood of Rabbits.†

Anti BSA N inj., μg‡	I* BSA N inj., μg	% of total inj. antigen remaining in blood	
		10 min.	24 hr
1	.18	8.5	1.5
None	.18	86	24.5

† Recorded as avg of results from 2 rabbits.

‡ Anti BSA combined with I* BSA during 30 min. incubation period *in vitro*.



Elimination of I* BSA from animals inj. with I* BSA-anti BSA complexes or I* BSA.
Fig. 1, rabbits. Fig. 2, guinea pigs. Fig. 3, mice.

pletely eliminated 10 minutes following injection.

Elimination of I antigen-antibody complexes from guinea pigs and mice.* Elimination of radioactivity from guinea pigs and mice injected intravenously with I* antigen-antibody complexes prepared *in vitro* at equivalence and from guinea pigs and mice injected with I* antigen alone is shown in Tables III and IV and in Fig. 2 and 3. Animals which received I* BSA-anti BSA complexes or I* BGG-anti BGG complexes showed a significantly more rapid decrease in total body radioactivity at 24 hours than animals which received I* BSA or I* BGG alone. Mice injected with I* BSA-anti BSA complexes prepared with 2.5, 5 and 10 times the amount of antigen necessary to precipitate all of the antibody at equivalence (2.5, 5 and 10 times equivalence) showed progressive decrease in rate of immune elimination of the I* BSA with increasing degree of antigen excess (Table IV). The mice receiving I* BSA-anti BSA complexes prepared with I* BSA at 10 times equivalence demonstrated rates of antigen elimination which approached rates of elimination in control mice which were injected with I* BSA alone.

TABLE III. Elimination of Both Free I* Antigen and I* Antigen-Antibody Complexes from Guinea Pigs.†

Antibody N inj., µg	Antigen N inj., µg	% of total inj. antigen remaining in whole animal			
		2 hr	5 hr	16 hr	24 hr
3 anti BGG	1 I* BGG	78	66	31	18
None	*Idem*	93	84	66	55
1.1 anti BSA	.2 I* BSA	86	67	26	14
None	*Idem*	96	88	64	54
.3 anti BGG	.1 I* BGG	94	68	32	16
None	*Idem*	93	85	64	53
.03 anti BGG	.01 I* BGG	78	69		18
None	*Idem*	98	92		49
.055 anti BSA	.01 I* BSA	85	72		26
None	*Idem*	96	94		75

† Recorded as avg of results from 2 animals.

TABLE IV. Elimination of Both Free I* Antigen and I* Antigen-Antibody Complexes from Mice.†

Antibody N inj., µg	Antigen N inj., µg	% of total inj. antigen remaining in whole animal			
		2 hr	5 hr	16 hr	24 hr
3 anti BGG	1 I* BGG	97	95	21	6
None	*Idem*	97	88	54	38
.3 anti BGG	.1 I* BGG	83	56	19	7
None	*Idem*	91	85	58	39
.055 anti BSA	.01 I* BSA	84			10
Idem	.025 I* "	86			22
"	.05 I* "	90			25
"	.1 I* "	93			31
None	.01 I* "	94			36

† Recorded as avg of results from 2 animals.

Discussion. Our results show that immune elimination of I* antigen can be used to detect the presence of trace amounts of antibody in passively immunized animals, animals injected with I* antigen-antibody complexes and, presumably, actively sensitized animals. The results were shown in passively immunized rabbits or in rabbits, mice and guinea pigs injected with I* antigen-antibody complexes. Rapid elimination of an appropriate amount of I* antigen resulted from passive sensitization of a rabbit with 1 μg of antibody N. In a 3 kg rabbit with a total plasma pool equal to 300 ml, amount of antibody N per ml of plasma detected was .0033 μg. Therefore, immune elimination may be detected in passively (and presumably actively) sensitized rabbits in which the antibody levels are as low as .0033 μg antibody N/ml of plasma.

The I* antigen-antibody complexes, containing a total of no more than .03 μg of antibody, were rapidly eliminated from guinea pigs as shown by more rapid decrease in whole body radioactivity. Thus, in a 500 g animal with a total plasma volume of 25 ml, .0012 μg of antibody N/ml of plasma should be detectable in a sensitized guinea pig.

The amounts of antibody used for passive immunization and for injection of I* antigen-antibody complexes were injected in a volume of 1 ml. Therefore, the sensitivity of the method can be expressed as μg of antibody N/ml of serum and was found to be from .0012-.0033 μg antibody N. However, if the same amount of antibody were present in a larger volume which is limited only by the amount which can be safely injected intravenously in a given species of animal, approximately 20 ml per rabbit or 5 ml per guinea pig, the relative sensitivity of the test as expressed by μg antibody N/ml of serum would be greatly increased.

The sensitivity of this method compares favorably with that of other methods of detecting antibody(15). For the quantitative precipitin reaction, the lower limits of detection are approximately 20 μg of antibody N.; for the hemagglutination reaction, .003 μg of antibody N/ml serum; for local passive cutaneous anaphylaxis, .03 μg antibody N/ml serum; for the complement fixation reaction, .1 μg antibody N/ml serum; and for passive anaphylaxis in the guinea pig, 10 μg of antibody N. The precipitation of I* antigen-antibody complexes by ammonium sulfate precipitation(14) is a method equally sensitive to that observed here, but this technic is limited to systems in which the free antigen is not precipitated by the method used to precipitate the antibody or the antigen-antibody complexes.

The rapid immune elimination technic cannot be used to make an absolute quantitation of antibody. However, the results in mice injected with antigen-antibody complexes prepared at varying antigen-antibody ratios indicate that the use of varying amounts of I* antigen could be employed to approximate the amount of antibody (Table IV). The maximal amount of I* antigen which could be injected and still be eliminated at an increased rate approximated the amount which would combine with the total circulating antibody at equivalence.

The level of antibody which can be detected by the immune elimination technic is limited by the smallest amount of I* antigen that can be traced *in vivo,* which is determined by the specific activity of the antigen. Specific activity of the antigen is limited by the amount of I* which can be conjugated to protein without chemical or radiochemical denaturation of protein. In our experience, using commercially available carrier-free I*, the limit of specific activity is approximately 2-3 μc per μg protein N.

Rabbits injected with 1.1 to 4.5 μg of antibody N and 1/10 of this amount of I* antigen retained considerably more I* antigen in their circulation after 10 minutes than rabbits injected with 22.5 and 45 μg of antibody N and proportionately the same amounts of I* antigen. Since in all anti BSA immunized animals tested, the I* antigen present in the blood was almost entirely in the form of antigen-antibody complexes after 10 minutes, it seems likely that the rabbits injected with the smaller amounts (1.1-4.5 μg) of antibody and antigen contained complexes which were of different composition than the antigen-antibody complexes formed in rabbits injected with the larger amounts (22.5-45 μg). Antigen-antibody complexes formed in rabbits injected with 1.1 and 4.5 μg of antibody N were probably diluted in the plasma to such a degree that they were smaller and removed more slowly by phagocytic cells than complexes formed by higher concentrations of antigen and antibody.

Elimination of antigens from rabbits passively immunized with 1.1-45.0 μg of antibody N bears on the natural selection theories of antibody formation. These theories postulate that the normal gamma globulins consist of a variety of molecular species(16), one or more of which by chance are likely to react with any given antigen. The effect of 1.1 μg of anti BSA per rabbit on elimination of I* BSA as compared with controls injected with I* BSA alone indicates that any natural anti BSA globulins must be present in amounts less than the 1.1 μg of antibody N per rabbit which was used for passive immunization. In addition, unsensitized normal rabbits failed to show evidence of a significant amount of circulating I* BSA-anti BSA complexes following injection of small amounts of I* BSA (Table I). Although a trace amount of I* BSA was precipitated with globulin 10 minutes following injection of I* BSA, this was apparently non-specific since a prior injection of a large amount of non-labeled BSA failed to decrease the amount of I* BSA precipitated with the globulins. If a 3 kg rabbit has approximately 3 g of gamma globulin, 1.1 μg of anti BSA N would be less than 1/400,000 of the rabbit's total gamma globulin. Thus, if natural globulins capable of combining with the various BSA antigenic determinants exist, they must total less than this fraction of the gamma globulin. Considering the .5 kg guinea pigs injected with antigen-antibody complexes containing .03 anti BGG N, this amount of antibody was equal to approximately 1/2,555,000 of the gamma globulin in the animal. Since this amount of antibody caused an immune elimination, any natural globulins capable of combining with the various BGG antigenic determinants would necessarily be present in an amount smaller than 2×10^{-7} g of antibody protein per animal. The levels of naturally occurring antibody against these serum protein antigens are considerably lower in the rabbit and guinea pig than would be expected if the gamma globulin were made up of a random mixture of 5,000(16) to 50,000(17) different molecular species. Therefore, if the gamma globulin of these species consists of mixtures of some such number of molecular species, the amounts of these different molecules must be extremely unequal.

Summary. Immune elimination of antigen using very small amounts of antigen and antibody occurs in characteristic fashion in rabbits, guinea pigs and mice and may be used as an additional sensitive method for demonstration of small amounts of antibody against serum protein antigens. Consideration of these findings in regard to the hypothesis of natural antibodies is discussed.

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Received Sept. 15, 1960. P.S.E.B.M., 1960, v105.

Degradation of Nucleotide Pyrophosphate Compounds by Human Seminal Plasma Enzymes.* (26102)

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The observation has been made that phosphate is relased from a variety of purine and pyrimidine containing nucleotide pyrophosphate compounds by combined action of nucleotide pyrophosphatase activities(1) and 5-nucleotidase(2) found in bovine seminal plasma. The present report indicates the presence of similar activities in human seminal plasma.

Materials and methods. Substrates were purchased from Sigma Chemical Co., St. Louis, Mo., except for yeast cytidylic acid, which was a Nutritional Biochemicals preparation. Pooled frozen human semen samples, 5 or more per pool, were obtained from the Duke Medical Center Fertility Clinic. The pooled samples were thawed and centrifuged at 10,000 × g for 15 minutes at 5° to remove sperm. The crude human seminal plasma was then fractionated by the short procedure used by Heppel and Hilmoe to prepare 5-nucleotidase from bovine seminal plasma(2), followed by precipitation with ammonium sulfate. The 0 to 67 and 67 to 80% saturated ammonium sulfate precipitates were dissolved in minimum amounts of water, and frozen until used. Phosphate was determined by the method of Gomori(3). Phosphate release is recorded as the difference between that amount found in zero time (unincubated) and final time (incubated at 37°) supernatants of 5% trichloracetic acid deproteinized, 0.2 ml reaction mixtures. Spectrophotometric activity measurements, using O-carboxyphenylphosphate and Ca(bis-pNO$_2$ phenyl phosphate)$_2$ as substrates, were carried out at room temperature according to procedures described by Hofstee(4) and Privat de Garilhe and Laskowski(5), respectively. Reaction mixtures contained 0.08 M glycine, pH 8.9, 10^{-2} M MgCl$_2$, enzyme, and substrate as indicated.

Results. The 2 ammonium sulfate fractions obtained were assayed at several concentration levels for 5-nucleotidase activity. Roughly two-thirds of total activity was found in the 0-67% ammonium sulfate fraction, which was then used for the experiments summarized in Table I. In addition to the substrates shown, crude seminal plasma and both enzyme fractions were assayed for activity against the phosphomonoesterase substrate, O-carboxyphenylphosphate and the phosphodiesterase substrate Ca(bis-pNO$_2$ phenyl phosphate)$_2$. Crude seminal plasma samples slowly hydrolyzed both substrates, but neither of the 2 ammonium sulfate fractions exhibited appreciable activity.

Discussion. The observations reported indicate that the "short" procedure of Heppel and Hilmoe(2) allows the preparation, from human seminal plasma, of 5-nucleotidase relatively free of "alkaline" phosphomonoesterase. The preparation also contains nucleotide pyrophosphate degrading activities, presumably nucleotide pyrophosphatases. It is interesting to note that the substrate spec-

TABLE I. Orthophosphate Release from Nucleotides by Human Seminal Plasma Enzymes.

Additions, μmoles		Phosphate released, μmoles
30 min. incubation		
Cytidine-5-phosphate	.24	.20
Uridine-5-phosphate	.25	.17
Adenosine-5-phosphate	.25	.15
Guanosine-5-phosphate	.25	.10
Yeast cytidylic acid	.25	.01
Glucose-1-phosphate	.20	.02
Phosphoryl-choline	.20	.02
Flavine mononucleotide	.20	.02
Inorganic pyrophosphate	.20	.00
60 min. incubation		
Cytidine diphosphate choline	.20	.14
Uridine diphosphate glucose	.34	.19
Diphosphopyridine nucleotide	.20	.22
Reduced diphosphopyridine nucleotide	.4	.30
Flavine adenine dinucleotide	.2	.24

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* Supported by research grant from Nat. Inst. of Allergy and Inf. Dis., N.I.H., U.S.P.H.S.

trum exhibited by the human seminal plasma enzyme preparation is similar to that of the bovine seminal plasma enzyme preparation made by the same procedure(1).

Summary. Enzymes prepared from human seminal plasma were found to release phosphate from the 5-mononucleotides, cytidylic, uridylic, adenylic and guanylic acids. The preparations were not, or only slightly, active in hydrolyzing yeast cytidylic acid, glucose-l-phosphate, phosphorylcholine, flavine mononucleotide, and O-carboxyphenyl phosphate. Phosphate was also released from cytidine diphosphatecholine, uridine diphosphate glucose, oxidized and reduced diphosphopyridine nucleotide, and flavine adenine dinucleotide.

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Received Sept. 21, 1960. P.S.E.B.M., 1960, v105.

Cholic Acid in Guinea Pig Bile.* (26103)

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During the investigation of formation of gall bladder precipitates produced by parenteral administration of a bacterial polysaccharide, we found that 3α, 7α, 12α-trihydroxycholanic (cholic) acid was not only present in guinea pig bile, but was the major bile acid. Further study disclosed that 3α, 7α, 12α-trihydroxycholanic acid was present in adult but not in immature guinea pigs, thereby offering an explanation for the previously reported absence(1,2) of this bile acid.

Methods. Non-inbred, albino and varicolored guinea pigs were obtained from commercial suppliers in Oregon, Utah, and Missouri. Eighteen adult guinea pigs of either sex, approximately 5 months of age and weighing 500-700 g, were fed with Purina rabbit chow and fresh green vegetables, carrots and apples *ad libitum*. Bile fistulae were performed on 6 animals from this group. Bile from the gall bladders of the remaining 12 animals was pooled in 2 samples. Seventy-two immature guinea pigs of either sex, 6-8 weeks of age, and weighing 250-350 g were divided into 3 equal groups. Each group of 24 animals was kept for 2 weeks on one of the following diets: a) Vit. C deficient diet of Rinehart and Mettier(3), rich in other vitamins, fats, and minerals; b) Purina rabbit chow heated for one hour at 100°C; c) Purina rabbit chow with green vegetables, carrots and apples as supplied to the group of adult guinea pigs. Bile from gall bladders was pooled in 3 samples per each dietary group. All gall bladders were aspirated by syringe within one minute after application of a neck clamp.

The bile fistulae were performed under ether anesthesia. One polyethylene catheter was inserted into the gall bladder and another one into the proximally ligated common bile duct. Both catheters were passed under the skin to the back where their free ends were pulled through a cutaneous wound and connected to allow enterohepatic cycling. To avoid operative and postoperative abnormalities, collection of the first bile samples was not begun until 48 hours after the surgical procedure. A volume of normal bile equal to that diverted was supplied through the duodenal fistulae. These samples obtained from the bile fistulae were not pooled but processed separately.

Except for small aliquots, the bile samples

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* Supported by research grant, Nat. Inst. Arth. & Met. Dis., U.S.P.H.S.

were acidified with hydrochloric acid, extracted with n-butyl alcohol, and submitted to partition chromatography on columns of kieselguhr treated with silane as described by Bergström and associates(4,5,6). The solvent system, isooctanol: chloroform (1:1) as stationary phase and 50% aqueous methanol as moving phase, was used for separation of the conjugated bile acids. For separation of the free bile acids the solvent system, chloroform: heptane (9:1) as stationary phase and 58% aqueous methanol as moving phase, was used. The free bile acids were obtained by hydrolysis of the conjugated bile acids in 1N NaOH for 5 hours at 110°C. The effluents were titrated with 0.01 N NaOH in methanol. Aliquots of original untreated bile, the butanolic extracts, and the conjugated and free bile acids obtained by column chromatography were submitted to paper chromatography in the systems introduced by Sjövall(7,8). After paper chromatography, the bile acids were detected by spraying the paper strips with phosphomolybdic acid(7). However, only 2 of the 3 bile acids separated by the chromatography on columns, gave a color reaction with this reagent. Therefore, the chromatograms were sprayed also with 15% phosphotungstic acid in absolute ethanol, as used by Martin(9) for detection of several sterols, but modified by heating at 80°C until development of pink spots within 3-5 minutes. All 3 bile acids separated on columns could be located by this procedure. After separation on the columns, the free bile acids were further purified as described by Bergström and Sjövall(6), and after several recrystallizations, were identified by infrared spectra, spectra in concentrated sulphuric acid(10), melting points, and specific rotations.

Results. Bile collected by fistula and from gall bladders of the mature guinea pigs contained 3*α*, 7*α*, 12*α*-trihydroxycholanic acid as the major bile acid, as well as 3*α*, 7*α*-dihydroxycholanic (chenodeoxycholic) and 3*α*-hydroxy, 7-ketocholanic (7-ketolithocholic) acid, with average ratio approximating 4:3:1. A typical separation of the conjugated and free bile acids by column chromatography is shown in Fig. 1.

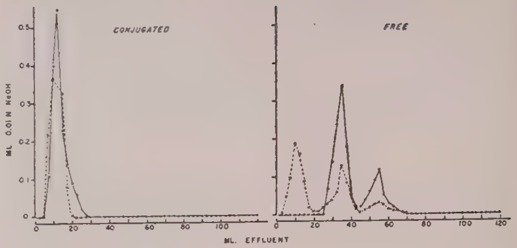

FIG. 1. Separation of bile acids by column chromatography. *– – – –* adult animals. ●———● immature animals.

In the immature guinea pigs 3*α*, 7*α*, 12*α*-trihydroxycholanic acid was not found in any of the bile samples, but the other 2 acids, 3*α*, 7*α*-dihydroxycholanic and 3*α*-hydroxy, 7-ketocholanic acid, were present in an approximate average ratio 2.5:1 (Fig. 1). The bile acid pattern in immature guinea pigs was the same in each of the 3 dietary groups.

In both mature and immature animals, the bile acids were found to be conjugated with taurine. No evidence was obtained by either column or paper chromatographic procedures that a portion of the bile acids was conjugated with glycine.

Bile acids were crystallized as follows: 3*α*, 7*α*, 12*α*-trihydroxycholanic acid: aqueous acetic acid (1×), aqueous methanol (2×); 3*α*, 7*α*-dihydroxycholanic acid: ethyl acetate (1×), ethyl acetate and petroleum ether (2×); 3*α*-hydroxy, 7-ketocholanic acid: aqueous acetic acid (1×), aqueous methanol (1×), ethyl acetate (2×). The infrared spectra corresponded to authentic samples of 3*α*, 7*α*, 12*α*-trihydroxycholanic (Fig. 2), 3*α*, 7*α*-dihydroxycholanic, and 3*α*-hydroxy, 7-ketocholanic acid. The values obtained for maximum absorption in concentrated sulphuric acid, melting points, and specific rotations (Table I) further identify these bile acids.

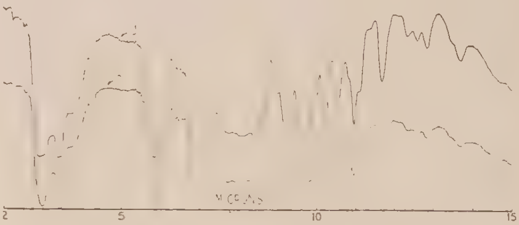

FIG. 2. Infrared spectrum of 3*α*,7*α*,12*α*-trihydroxycholanic acid isolated in this study (U) and of an authentic sample (S).

TABLE I. Absorption Maxima in Conc. H_2SO_4, Melting Points and Specific Rotations of the 3 Bile Acids Isolated in This Study Compared with References.

	3α,7α,12α-trihydroxy-cholanic acid		3α,7α-dihydroxy-cholanic acid		3α-hydroxy, 7-ketocholanic acid	
	Isolated	Reference	Isolated	Reference	Isolated	Reference
Absorb. max., mu	389	389(10)*	310	310(10)	301	301(10)
M. P., °C	199	199(15)	143	143(15)	198–201	201–203(1)
$[\alpha]_D^{25°}$ (Ethanol)	+37 ± 1.5	+37(15)	+11 ± 0.5	+11.5(15)	–27 ± 1.0	–27.27(1)

* Reference.

Discussion. There are 2 general aspects of bile acid formation: a) the characteristic pattern of bile acids of a species reflects phylogenetic development(11) and b) change in bile acids during enterohepatic cycling with conversion of trihydroxycholanic acids by intestinal microorganisms and with hepatic reconversion in some species(12). The abundance of 3α, 7α, 12α-trihydroxycholanic acid and the conjugation of all bile acids with taurine found in the present investigation, would characterize the guinea pig, at least the adult of this species, as less "modern" phylogenetically than has been thought(13).

The observation that 3α, 7α, 12α-trihydroxycholanic acid appears in the bile of the guinea pig only upon maturation is not explained through any known mechanism. It is possible that a similar effect of aging may be found in other species. Danielsson and Kazuno's(2) failure to find 3α, 7α, 12α-trihydroxycholanic acid in the guinea pig may be explained by the immaturity of their animals. Weights or age of the guinea pigs used by Imai(1) were not reported. It is of interest that Iwaki(14) observed cholic acid in the urine of guinea pigs given pentahydroxybufostane.

In our unpublished studies on conversion of cholesterol-4-C^{14} to bile acids in the guinea pig, labeled 3α, 7α, 12α-trihydroxycholanic acid appeared in the bile more slowly but attained higher concentration than 3α, 7α-dihydroxycholanic and 3α-hydroxy, 7-ketocholanic acid. These studies also suggest that the 3 bile acids form without action of enteric microorganisms in the guinea pig but that 3α, 7α, 12α-trihydroxycholanic acid may slowly accumulate in the adult animal by preferential conversion and recycling. However, such a mechanism would not explain the apparent absence of 3α, 7α, 12α-trihydroxycholanic acid in the bile of the immature guinea pig.

Summary. Cholic (3α, 7α, 12α-trihydroxycholanic) acid has been isolated from the guinea pig bile obtained from gall bladders as well as by biliary fistulae. However, this bile acid was found only in the bile of adult and not of immature guinea pigs. The other 2 bile acids isolated, chenodeoxycholic (3α, 7α-dihydroxycholanic) and 7-ketolithocholic (3α-hydroxy, 7-ketocholanic) acid, occurred in both immature and mature guinea pigs.

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Received Sept. 6, 1960. P.S.E.B.M., 1960, v105.

A New Bovine Adenovirus Related to Human Adenovirus.[*] (26104)

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We have previously reported on isolation of a bovine adenovirus, type unknown, that is antigenically related to a human adenovirus (1). The initial demonstration that the Bovine virus #10 was related to some human virus was based upon neutralization of the agent by human gamma globulin and individual human sera. Identification of the agent as an adenovirus was based upon its growth properties and presence of the group specific adenovirus complement fixing antigen. The bovine virus was not neutralized by antisera to adenoviruses types 1-7 and we do not know whether it is related to some other known type or an unidentified human adenovirus. The demonstration by Gold and Ginsberg(2) that neutralizing substances to many human adenoviruses could be found in cattle suggested that other adenoviruses would be isolated from cattle. This paper reports a second bovine adenovirus that is related to an unidentified type of human adenovirus.

Materials and methods. The Bovine virus #19 was isolated in June, 1956 from the feces of an apparently well calf maintained as part of the dairy herd at Glen Mills School in Pennsylvania. Procedure for collecting, processing, and isolating these viruses has been previously described(1). Briefly, trypsinized calf kidney cells were used for isolation of the agent. Neutralization tests to determine the possible relationship of the bovine virus to some human agent were carried out with human adult sera and several lots of human gamma globulin. Complement fixation tests confirming Bovine #19 as an adenovirus were kindly carried out by Dr. Hummeler of the Children's Hospital.

Results. The virus on initial isolation gave a slight cytopathogenic effect (CPE) after 13 days incubation. After several passages the CPE was observed in 5-6 days though total destruction of the cells was rarely observed. The titer has remained low and 0.1 ml of virus was never infectious for calf kidney cells at dilutions greater than 10^{-3}. The CPE is shown in Fig. 1. The virus gives the characteristic range of adenovirus intranuclear inclusion bodies. The nucleus is often enlarged and there are a variety of patterns of nuclear changes similar to those described for human adenoviruses(3). We have obtained growth of this virus only in trypsinized calf kidney cells and no CPE has been observed either in HeLa cells or monkey kidney cells which distinguishes this bovine agent from a human adenovirus. No detectable growth of the virus was observed in adult or suckling mice, chick embryos (all routes), guinea pigs, hamsters, or rabbits. No hemagglutination was observed with red cells from the guinea pig, sheep, cow, chicken, or human. Rat and mouse red blood cells were agglutinated by Bovine #19. One-half ml of a 1:64 dilution of virus agglutinated 0.5 ml of a 0.5% suspension of rat and mouse blood cells after 4 hours incubation at 4°C. Nonspecific hemagglutination was not observed with the maintenance medium in which the cells were grown. By contrast the Bovine adenovirus #10 agglutinates rat but not mouse red blood cells. This specificity in agglutination of rat and mouse red blood cells by strains of human adenoviruses has

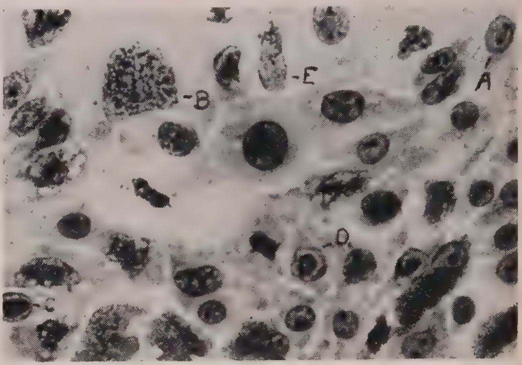

FIG. 1. Bovine #19 grown in calf kidney cells, 4 days, May-Greenwald-Giemsa. A. Normal nucleus. B, C, D, E. Enlarged nuclei with varying patterns of chromatin clumping.

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[*] Supported by U.S.P.H.S. grant.

TABLE I. Neutralization of Bovine Virus #19 by Sera of Adults and Infants.

	No. tested	Neg (1:4)	Positive 1:4	1:8	1:16	1:32	1:64	% positive
Adult sera *vs* 100 TCD	50	41 (82%)	2	1	1	2	3	18%
Infants' sera *vs* 100 TCD	25	25						0%

Age of infants ranged from 4-15 mo. All sera heated to 56°C for 30 min.

been described by Rosen(4). Identifying properties of Bovine #19 are, then: 1) characteristic intranuclear inclusion bodies, 2) growth in trypsinized calf kidney cells to low titer, and 3) hemagglutination of rat and mouse blood cells.

The initial Bovine adenovirus isolate #10 and the present Bovine #19, though very similar in growth properties, differ not only in hemagglutination of mouse red blood cells but antigenically. There is no antigenic cross reaction between these agents. A 1:320 dilution of sera from rabbits immunized with Bovine #10 neutralized 100 TCD of the homologous virus but did not neutralize 10 TCD of Bovine #19 at a dilution of 1:20. Under similar conditions antisera prepared against Bovine #19, and also active at a 1:320 dilution, failed to neutralize 10 TCD of Bovine #10 at a 1:20 dilution.

Identification of Bovine #19 as a virus related to a human adenovirus. Bovine #19 was recognized as being related to some human virus because 100 TCD of virus were neutralized by a 1:40 dilution of human gamma globulin. This human relationship was confirmed by showing that 18% of human adult sera and no infant sera neutralized the agent (Table I). Bovine #19 was specifically identified as an adenovirus by the complement fixation test. The virus grown in calf kidney cells was used as a live preparation to immunize guinea pigs and hamsters. Their antisera were tested against a human adenovirus grown in HeLa cells and the results are shown in Table II. Sera from both guinea pigs and hamsters immunized with Bovine #19 gave positive complement fixation titers with human adenovirus antigens. We were not able to prepare a satisfactory complement fixing antigen of Bovine #19 that could be used against human sera. Whether Bovine #19 is antigenically related to a known type of human adenovirus or an as yet unidentified human adenovirus remains to be determined. Our agent was not neutralized by antisera against types 1-7 adenoviruses.

TABLE II. Reaction of Antisera to Bovine #19 with Complement Fixing Antigen of Human Adenovirus.

Bovine #19 antisera	C.F. antigen	C.F. titer
Guinea pig		
Pre-immunization sera	Human adenovirus	<1:8
	Tissue control*	<1:8
Post- " "	Human adenovirus	1:8
	Tissue control	<1:8
Hamster		
Pre-immunization sera	Human adenovirus	<1:8
	Tissue control	<1:8
Post- " "	Human adenovirus	1:16
	Tissue control	<1:8

* Guinea pigs and hamsters immunized with virus grown in calf kidney. C.F. adenovirus antigen grown in HeLa cells. Tissue control = HeLa cells alone used as antigen.

Confirmation of bovine origin of adenovirus. Since Bovine #19 is antigenically related to a human adenovirus additional studies were carried out to confirm that this was not a human laboratory contaminant but a bovine virus antigenically related to a human adenovirus. As noted above, the virus did not grow in HeLa cells or monkey kidney cells, but grew in continuous passage in freshly trypsinized calf kidney cells, a property which distinguishes it from a human adenovirus. Further, the agent was reisolated from the original fecal specimen. Sera obtained from the cow before viral isolation failed to neutralize the agent, while sera obtained 9 weeks after isolation of the agent neutralized 100 TCD of the virus at a dilution of 1:20. The agent is a common bovine virus, for all of 40 cows had neutralizing antibodies to the virus (Table III). The results confirm the status of Bovine #19 as a widely distributed bovine adenovirus antigenically related to or identical with an unknown type of human adenovirus. Though Bovine #19 has not yet been inoculated into cattle, Bo-

TABLE III. Incidence of Neutralizing Antibodies in Adult Bovine Sera to Bovine Virus #19.

No. tested	Serum dilution *vs* 100 TCD		Total positive
	1:16+	1:4	
40	Pos 35 (88%)	Pos 5 (12%)	40 (100%)

vine #10 has. Dr. James Gillespie, Veterinary Virus Research Inst., Cornell Univ., using our fourth bovine kidney tissue culture passage, inoculated intranasally 10 ml of a 1:10 dilution of virus into 2 susceptible animals. Neither animal showed any evidence of clinical illness, though both animals were infected as indicated by a rise in titer in the neutralization test from less than 1:4 to 1:32 and 1:128 respectively.

Discussion. In their original observations on presence of neutralizing substances to human adenoviruses in bovine sera, Gold and Ginsberg(2) refrained from interpreting the nature or origin of these neutralizing substances, though studies on the nature of the inhibitor were consistent with its being an antibody. In a discussion of the nature of neutralizing substances in animal sera(5), we concluded that inhibitors such as the reported adenovirus inhibitors are true antibodies resulting from viral infection. Isolation of 2 bovine adenoviruses related to human adenoviruses allows one to conclude that the neutralizing substances to the many human adenovirus types in bovine sera are antibodies to bovine adenoviruses antigenically related to human adenoviruses. As noted above, neither of our bovine adenoviruses have been typed, though they are apparently not related to adenoviruses types 1-7. It is of interest to note that neutralizing antibodies to the important human types 4 and 7 are commonly present in bovine sera and the viruses responsible for these antibodies should ultimately be found in cattle.

Summary. A second bovine adenovirus has been isolated. The virus is antigenically similar to a human virus as determined by 1) neutralization with human gamma globulin and human adult sera, and 2) possession of a complement fixing antigen similar to that of the human adenovirus. The related type of human adenovirus remains unknown.

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Received June 13, 1960. P.S.E.B.M., 1960, v105.

Immunologic Mechanism of Anaphylaxis. III. Inhibition Phenomenon in Passive Cutaneous Anaphylaxis in the Mouse.* (26105)

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It has been shown that mouse skin can be passively sensitized to an immediate type of hypersensitive reaction(1,2,3). This type of reaction, which consists of an increase in permeability of the small vessels of the skin when antigen reacts with antibody, has been termed "passive cutaneous anaphylaxis" (PCA)(4,5). In the guinea pig as in the mouse, a latent period is required between intradermal injection of antibody and intravenous challenge with antigen(6,1). Benacerraf and Kabat have shown that a latent period is also required for passive systemic anaphylaxis(7). This requisite latent period is evidence that a fixation of the antibody to the tissues occurs before the reaction is elicited.

Further evidence that fixation of antibody

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* This investigation was supported in part by grant from U.S.P.H.S., No. E-3075.

occurs was furnished by the demonstration that normal rabbit serum competitively inhibits passive cutaneous anaphylaxis in the guinea pig by competing with the antibody for fixation sites(8). Prochazka-Fisher and Cooke confirmed this observation and showed that the globulin fraction was responsible for this inhibition(9). Biozzi, Halpern and Binaghi confirmed and, by quantitative data, extended these observations(10).

The latent period in the mouse, however, was shown to be very short(1,2). Therefore, the question arises as to whether fixation in the mouse does, in fact, exist. A very good way to investigate this query is to see whether rabbit gamma globulin competitively inhibits passive cutaneous anaphylaxis in the mouse, as was demonstrated in the guinea pig. The present study investigates this possibility.

Materials and methods. Animals. Male, adult albino mice weighing approximately 20-25 g were used. *Antibody.* A pool of rabbit anti-egg albumin sera (anti-EA) (505 μg antibody N/ml) was employed. *Rabbit gamma globulin.* A purified rabbit gamma globulin (RGG) (Pentex, Lot 63 FO 5) was used and was diluted in 0.15 M NaCl to a concentration of 10 mg protein/ml and was kept frozen until used. *Antigens.* Twice recrystallized egg albumin (Armour) (EA) was used as antigen. *Histamine Dichlorhydrate.* A stock solution of 250 μg/ml (Fisher, Lot 542851) was prepared in 0.15 M NaCl and diluted 1/50 (2.5 μg/0.05 ml) just prior to use. *Diluent.* 0.15 M NaCl was used for all dilutions, except when the RGG solution was used. *Dye.* Evans blue (Eastman Organic Chemicals), 0.5% was prepared and stored at room temperature until used. The serum was diluted in 0.15 NaCl or in the RGG solution. The volume used for all intradermal injections was 0.05 ml. All intradermal injections were performed on the freshly shaved dorsal surface of each mouse using a short bevel, 26-gauge needle. Care was taken to inject the inoculum only into dermal layers of the skin with as little irritation as possible, to avoid non-specific traumatic reactions. Four intradermal injections were made on the back of each mouse.

In preliminary experiments, it was seen that minimal amount of antibody necessary to produce a reaction graded 3+ or more, in the mouse, was approximately 0.15-0.30 μg antibody N with this system.

Simultaneously with intradermal injection of antibody, 2 other sites were infiltrated, one with saline and the other with RGG. These injections sites were marked so that immediately after challenge with antigen, histamine could be injected in the same sites.

A latent period of one hour was allowed, after which time the specific antigen (EA) was injected intravenously with the dye. Intravenous injections were executed with a sharp, 27-gauge needle through the dorsal vein of the penis, after light ether anesthesia. The volume used in all injections was 0.25 ml. The antigen was mixed with an equal volume of dye before injection and either 0.3, 0.6, or 1.25 mg EA/0.25 ml was injected. Various antigen and antibody concentrations were used because it was found difficult to produce inhibition in this system.

The animals were killed one hour after challenge with antigen. They were skinned and the reactions (*i.e.,* accumulation of dye at site of intradermal injection) were read according to the diameter of the blue spots as follows: 0 = no reaction; ± = less than 4 mm; 1 = 4 mm; 2 = 4-6 mm; 3 = 6-10 mm; 4 = more than 10 mm.

Results. It is known that there is an inverse relation between amount of antibody and antigen used; that is, minimal amount of antigen which gives the maximal reaction is less when higher amounts of antibody are used(6). Moreover, it has already been pointed out by Osler, Hawrisiak, Ovary, Siqueira, and Bier, in a study on the mechanisms of hypersensitivity phenomena, that inhibition is much easier to obtain with threshold amounts of antigen(11). In preliminary experiments it was seen that scarcely any inhibition was produced when the animals were injected with 0.3 μg antibody nitrogen and challenged with 1.25 mg EA. On the other hand, the animals injected with 0.15 μg antibody nitrogen and challenged with 0.3 or 0.6 mg Ea reacted very poorly.

Therefore, inhibition produced by RGG

TABLE I. Competitive Inhibition Produced by RGG in Passive Cutaneous Anaphylaxis Elicited by Anti-EA in the Mouse.

Part I	Degree of reaction in diameters*											
	Challenge dose of antigen											
Injections	.3 mg EA						.6 mg EA					
.3 μg N anti-EA in saline	0	4	3	3	4	3	4	4	4	4	4	4
Idem in RGG (10 mg/ml)	0	0	3	0	0	0	4	3	0	0	0	0
Histamine (2.5 μg) + saline	4	4	4	4	4	4	4	4	4	4	4	4
Idem + RGG (10 mg/ml)	4	4	4	4	4	4	4	4	4	4	4	4
Part II												
	1.25 mg EA											
.5 μg N anti-EA in saline	3	0	3	3	3	4						
Idem in RGG (10 mg/ml)	0	3	0	0	0	0						
Histamine (2.5 μg) + saline	4	4	4	4	4	4						
Idem + RGG (10 mg/ml)	4	4	4	4	4	4						

* See *Materials and methods* for key to diameters as represented by numbers in the Table. Vertical columns contain values for reactions in 4 inj. sites on a single mouse.

was investigated in 2 series of animals, one injected with 0.3, the other with 0.15 μg antibody nitrogen. The first series was challenged with 2 concentrations of antigen (0.3 and 0.6 mg EA protein/0.25 ml) and the second with only one concentration (1.25 mg EA protein/0.25 ml).

Five out of 6 mice receiving 0.15 μg antibody N/0.05 ml anti-EA and 1.25 mg EA showed complete inhibition by RGG (Table I, Part II).

Using 0.3 μg antibody N anti-EA, 9 of the 11 mice showed complete inhibition by RGG (Table I, Part I) with 0.3 or 0.6 mg EA. No inhibition of the reaction produced by histamine was observed.

Therefore, inhibition of passive cutaneous anaphylaxis in the mouse by RGG may be produced. However, the competition by RGG is not very strong, as was shown by the fact that adequate amounts of antibody and antigen had to be used to show good inhibition. The fact that competition by RGG in the mouse is very weak is not surprising, since rabbit antibody persists only for a short time at injected site(1,2).

Similar observations were made using another antigen-antibody system, *i.e.*, BGG and rabbit anti-BGG.

A possible explanation for this short persistence of antibody and poor inhibition by RGG might be that other factors play an important role in passive cutaneous anaphylaxis in the mouse. In systemic anaphylaxis in the mouse, for example, Burdon and Treadwell have shown that circulating complexes play an important role(12,3) whereas they do not in systemic anaphylaxis in the guinea pig.

Summary. 1) Competitive inhibition by RGG in passive cutaneous anaphylaxis in the mouse has been demonstrated. 2) Inhibition of PCA was much weaker than in the guinea pig. 3) No inhibition of reactions produced by intradermal injections of histamine was demonstrated. 4) The possible meaning of results was discussed.

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Received June 20, 1960. P.S.E.B.M., 1960, v105.

Effect of a Pseudomonas Fraction on Oxidase Activity of Mitochondria and Monocytes.[*] (26106)

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Berk *et al.*[†](1) indicated that the succinoxidase activity of cells obtained from mouse peritoneal exudates, as well as of homogenates of liver and spleen, was inhibited in presence of heat-killed *Pseudomonas aeruginosa*. Succinoxidase activity was also reduced when these cells (monocytes) were allowed to phagocytize dead Pseudomonas *in vivo*. Cytochrome oxidase activity of monocytes and tissue homogenates seemed to be less inhibited by the presence of dead organisms. Recent data obtained in this laboratory indicate that *in vitro* succinoxidase activity of mouse liver mitochrondria and of monocytes is also depressed by trace amounts of Piromen[‡] (a Pseudomonas polysaccharide).

Materials and methods. Mitochondria were prepared by homogenizing livers of 20 mice in chilled 8.5% sucrose-0.1 M phosphate buffer solution, pH 7.3, in a Waring blendor for 60 seconds. Following removal of cellular debris by low speed centrifugation, the homogenates were centrifuged for 20 minutes at 20,000 × g and the sedimented mitochondria resuspended in buffered sucrose solution. Peritoneal exudates (monocytes) were induced in 40 mice by injection of 0.5 ml mineral oil. The cells were harvested 16 hours later, washed, and examined manometrically as previously described(1). Manometric experiments were run in the conventional manner at pH 7.3 and total flask volume was 3.2 ml. The pH of the Piromen was adjusted to 7.3 with Na_2HPO_4 prior to use. Monocytes and mitochondria were incubated respectively with Piromen 30 minutes prior to addition of substrates. The incubation conditions for mitochondria studies were as follows: 20 μmoles of succinate or 50 μmoles of para-phenylenediamine (p-PDA), 0.4 mg N/ml of liver mitochondria, 100 μmoles of phosphate, pH 7.3; 0.5 μmole of cytochrome c, 2 μmoles of $MgCl_2$, 10 μmoles of NaCl. Monocyte experiments were performed with heparinized phosphate buffer, pH 7.3. Substrate and cytochrome levels were the same as in mitochondria experiments. All experiments were at 38°C.

Results. Table I indicates that both succinic and cytochrome oxidase activities of liver mitochondria were inhibited by as little as 3 μg of Piromen per ml. In one preparation, succinoxidase activity was completely abolished by 3 μg of Piromen per ml. It was more typical, however, for inhibition of succinoxidase activity to disappear upon prolonged incubation. Fig. 1 depicts this reversal of inhibition and indicates that the inhibitory effect of Piromen was greatest at beginning of reaction. The data in Table I and Fig. 2 suggest that cytochrome oxidase was more sensitive to Piromen inhibition than was succinoxidase. Although the greatest inhibition of cytochrome oxidase also occurred at beginning of reaction, nevertheless there was less reversal of inhibition than noticed in the succinate system. Since endogenous respiration of mouse liver mitochondria was negligible, it was difficult to deter-

TABLE I. Effect of Piromen on Succinic and Cytochrome Oxidase Activities of Mouse Liver Mitochondria.

		μl O_2/90 min.	
		Piromen*	
Substrate	Enzyme conc., mg N/ml	—	+
Succinic acid	.40	199	175
" "	.40	194	168
" "	.38	184	125
p-PDA	.55	293	216
"	.40	243	170
"	.40	237	168

* 3 μg/ml.

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[*] This investigation supported by grant from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

[†] Presented at joint meeting of the Southern Calif. Branch of Soc. Am. Bact. and Western Branch of AAAS, San Diego, June 17, 1959.

[‡] Travenol Labs., Morton Grove, Ill.

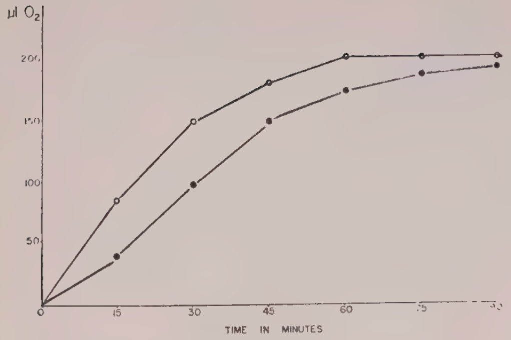

FIG. 1. Effect of Piromen on succinic oxidase activity of liver mitochondria. Open circles represent activity in absence of Piromen; closed circles represent activity in presence of 5 µg of Piromen/ml.

mine whether this activity was sensitive to Piromen.

Kerby(2) previously reported that Piromen disrupts the cell structure of human leukocytes. Therfore, Piromen-treated monocytes were also included in this study. The data in Table II indicate that the succinoxidase activity of mouse monocytes was sensitive to Piromen and degree of inhibition was proportional to concentration of polysaccharide added. However, the cytochrome oxidase activity of several preparations examined appeared to be relatively insensitive to inhibition. Where inhibition was observed with cytochrome oxidase there was no good correlation between degree of inhibition and Piromen concentration. It should be emphasized that the inhibitory effect of Piromen on monocyte succinoxidase activity was not overcome with continued incubation as noted with liver mitochondria. Consequently, inhibition was not rate limiting, but rather suppressive.

TABLE II. Effect of Piromen Concentration on Succinic and Cytochrome Oxidase Activities of Mouse Monocytes.*

	µl O$_2$/120 min.	
Piromen, µg/ml	Succinate	p-PDA
.0	112	140
.5	110	
1.0	103	149
2.0		143
3.0	86	126
5.0	20	

* 10^7 monocytes/ml.

Since Piromen was inhibitory in trace amounts and thus resembled exotoxins in activity, the question arose as to its heat lability. Table III demonstrates that the ability of a solution of Piromen to inhibit mitochondria and monocytes is not reduced by boiling directly in the flame of a burner for a few seconds.

TABLE III. Comparison of Heated and Unheated Piromen on Succinic and Cytochrome Oxidase Activities of Mouse Cell Preparations.

Preparation	Substrate	µl O$_2$
Monocytes*	Succinate	177
	" + 5 µg Piromen/ml	21
	" + 5 µg heated Piromen/ml	46
Mitochondria†	p-PDA	346
	" + 3 µg Piromen/ml	262
	" + 3 µg heated Piromen/ml	238

* 10^7 monocytes/ml—180 min. incubation.

† .4 mg N/ml of liver mitochondria—90 min. incubation.

Discussion. Previous studies(1) indicated that the succinoxidase activity of either mitochondria (liver) or intact cells (monocytes, HeLa) could be depressed by heat-killed *Pseudomonas aeruginosa* or sonic fractions thereof. Glycolysis was not inhibited (un-

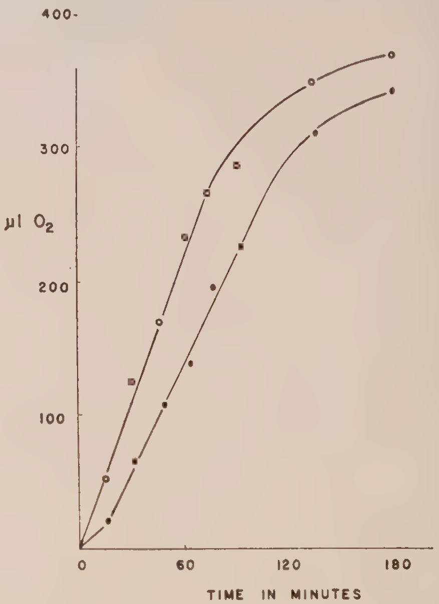

FIG. 2. Effect of Piromen on cytochrome oxidase activity of liver mitochondria. Open circles represent activity in absence of Piromen; closed circles represent activity in presence of 5 µg of Piromen per ml.

published results). In the present study inhibition of both succinic and cytochrome oxidases has been effected with a carbohydrate fraction of *Pseudomonas* (prepared commercially as Piromen). A few micrograms of Piromen were as inhibitory as several hundred micrograms N/ml of intact *P. aeruginosa,* and inhibition was proportional to concentration of Piromen used. With large amounts (5-10 μg) succinoxidase activity of monocytes was completely suppressed, but with lesser amounts only rate of reaction was affected.

Although both succinic and cytochrome oxidases were inhibited by Piromen, type and degree of inhibition was not constant for all tissues. In mouse monocytes succinoxidase activity could be reduced or completely abolished in proportion to amount of drug added. Once inhibited, little or no recovery of the system occurred. The cytochrome oxidase of monocytes was essentially resistant to inhibition. On the other hand, in liver mitochondria the succinoxidase system was able to overcome an initial inhibitory effect of Piromen, while the cytochrome oxidase system was more sensitive to inhibition by the drug. The fact that trace amounts of Piromen are toxic to cells and that the toxicity is heat stable suggests that Piromen possesses properties common to both exo and endotoxins.

The observation that mammalian oxidase activity is inhibited by trace amounts of a bacterial polysaccharide resembles that of Ajl(3) and Packer *et al.*(4). These investigators noted that alpha-keto glutarate oxidation in crude mouse liver homogenates and preparations of rat heart sarcosomes was inhibited by *Pasteurella pestis* toxin.

Piromen has been reported to be a nonprotein polysaccharide complex containing lipid and nucleic acids(5). It, therefore, differs chemically from Pasteurella toxin. Whether at the molecular level the active portions of the Pseudomonas and Pasteurella inhibitors differ or are very similar remains to be determined. In any case, some progress has been made toward determining the site of metabolic interaction between these parasites and their hosts.

In a related study, Gelzer and Suter(6) have reported that virulent strains of some bacteria, when phagocytized, inhibit endogenous respiration of rabbit leucocytes, whereas avirulent strains do not. The difference between virulence and avirulence may well be related to chemical differences in surface components (antigens) of the bacteria. Apparently bacteria differ with respect to site of their action on host cell metabolism, and although Pseudomonas and Pasteurella affect oxidative reactions, preliminary experiments with fractions of other organisms such as *Malleomyces pseudomallei, Vibrio fetus, Escherichia coli,*§ *Serratia marcescens,*§ and Salmonella typhose§ indicate no such activity.

Summary. A commercially prepared polysaccharide (Piromen) from a species of *Pseudomonas* inhibited both succinic and cytochrome oxidases of mouse liver mitochondria *in vitro.* Piromen alters rate of reaction rather than completely suppressing it. Cytochrome oxidase activity seemed to be more sensitive to Piromen than succinoxidase activity. Similar studies utilizing mouse monocytes indicated that succinoxidase activity could be reduced or completely abolished, but cytochrome oxidase activity was relatively insensitive to Piromen inhibition. Inhibition of monocyte succinoxidase activity was proportional to concentration of Piromen used.

The excellent technical assistance of Mr. John Meacham is gratefully acknowledged.

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Received June 20, 1960. P.S.E.B.M., 1960, v105.

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§ Difco.

Use of Amine Buffer (THAM) in Treatment of Acute Salicylate Intoxication.* (26107)

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2-amino-2-hydroxymethyl-1, 3-propanediol or tris (hydroxymethyl) aminomethane (THAM), an organic buffer of low toxicity, has been widely used for correction of metabolic and respiratory acidosis(1,2,3). In both conditions, a marked diuresis of alkaline urine was observed. Since an alkaline urine enhances excretion of weak acids(4,5,6,7), it was postulated that THAM would increase renal excretion of salicylic acid following salicylate intoxication. A simultaneous correction of the metabolic acidosis which accompanies salicylate poisoning was another expected result of this therapy.

Methods. Four mongrel dogs kept on a regular diet and given free access to water before the experiment were anesthetized with 30 mg/kg B. Wt of sodium pentothal injected intravenously. Indwelling catheters were introduced into both femoral veins and arteries and urinary bladder. An intravenous infusion of isotonic sodium chloride was administered to supply approximately 3% of body weight in 30 minutes, and to produce a measurable diuresis. Priming doses of inulin and para-amino-hippurate were injected intravenously to determine glomerular filtration rate and renal plasma flow. These doses were followed by a constant intravenous infusion calculated to replace the excreted amounts of these substances. Twenty minutes were allowed to permit an even distribution of the infused inulin and PAH. The urinary bladder was then emptied by injection of air and suprapubic pressure, and urine collections were made at regular intervals; at approximately mid-time of urine collection, blood was drawn from a femoral vein. After one or 2 control periods of 10 minutes, a single dose of 100 mg/kg sodium salicylate was injected intravenously. Thirty minutes later 0.3 M THAM solution was infused simultaneously with inulin and PAH. Rate of THAM administration was 1 ml/kg/min for the first 15 minutes and 0.5 ml/kg/min for the last 15 minutes. Total dose of THAM administered was 500-800 mg/kg. Inulin and PAH infusion were continued for an additional hour. Urine collections were made at 15, 30, 60 and 90 minutes after start of THAM administration. Blood pressure and ECG were monitored at regular intervals. Each sample of urine and plasma was analyzed for total salicylates, inulin, PAH, sodium, potassium, chloride, pH, CO_2, pCO_2 and HCO_3. pH was measured within 0.005 unit. The experimental procedure was designed as described by Kaplan and Del Carmen(4). Total salicylates in blood and urine were determined by the methods described by Brodie, Udenfried and Coburn(8) and by Smith, Gleason, Stoll and Ogorzalek(9). Determination of inulin was done by the Resorcinol method(10) and PAH by the method described by Smith *et al.*(11). Specimens were analyzed for their concentrations of sodium and potassium with the Patwin Flame Photometer and for chloride with the Buchler Cotlove chloridometer(12). For determination of pH the Astrup ME-1 micro equipment with the Sanz electrode was used. The microgasometer of Kopp-Natelson was employed for determination of plasma CO_2 content; HCO_3- and pCO_2 were calculated from the Henderson-Hasselbalch equation (13).

Results. Tables I and II present results of pH, CO_2 content, salicylate and electrolyte concentration in urine and plasma during the 4 experiments. Fifteen minutes follow ig onset of THAM infusion, salicylate excretion in the urine increased from 723 to 2838 μg/min a 400% increase, while pH of urine rose from 7.14 to 7.59. In contrast, be-kg) and Treatment with THAM. THAM was given at rate of .3 mM/kg for 15 min. and at rate of .15 mM/kg for next 15 min. Sample 1 is the control, samples 2 and 3 were taken 15 and 30 min. following salicylate admin.; samples 4 and 5 were measured during THAM admin.; 6 and 7 were taken 30 and 60 min. after THAM admin. Urine collections were made over entire length of each period. First figure in each column is avg of measurements. Figures in parentheses indicate range.

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* This work was partially supported by grant from N.I.H.

Period	Time, min.	Salicyl, μg/min.	Na^+	K^+	Cl^-	HCO_3^-	Urine output, ml/min.	GFR, ml/min.	pH	CO_2, μM/l	pCO_2, mm Hg
			μeq/min.								
1. Control		0	290 (217–403)	51 (28–96)	614	11.4	2.05 (1.11–4.00)	61	7.05 (6.88–7.23)	39 (13–65)	37.6 (31.0–44.1)
Salicyl inj.→	0										
2.	15	593 (430–871)	413	35	—	23.8	3.48	62	6.88	28	60.7
3. THAM infusion	30	723 (472–860)	342 (76–604)	47 (31–219)	1030	70.8 (61–81)	3.41 (2.45–5.55)	60	7.14 (6.9–7.42)	76 (67–85)	45.7 (39.1–52.2)
4.	45	1991 (1030–3670)	356 (37–835)	77 (41–104)	970	174.5 (155–195)	4.70 (2.74–7.4)	53	7.4 (7.2–7.67)	182 (165–198)	56.9 (51.0–62.8)
5.	60	2838 (1243–4824)	446 (61–837)	131 (61–189)	1000	152.5 (39–266)	7.65 (3.41–10.2)	42	7.59 (7.51–7.67)	341 (272–410)	59.8 (50.1–69.4)
6.	90	2076 (748–3519)	477 (117–770)	75 (7–128)	865	540	6.78 (4.78–10)	31	7.53 (7.48–7.62)	554	52.9
7.	120	2783 (2531–3035)	477 (144–885)	56 (19–78)	884	700	7.08 (5.23–8.60)	44	7.46 (7.37–7.55)	456	60.5

fore THAM administration, urine salicylate excretion increased from 593 to 723 μg/min, a 12% increase, while urine pH increased from 6.88 to 7.14. There was a 10-fold increase in HCO_3– excretion, while Na+ rose from 413 to 477 μEq/min and K+ from 47 to 131 μEq/min. Chloride excretion decreased from 1030 to 865 μEq/min. There was a marked diuresis with no significant changes in inulin or PAH clearance. Seventy-five minutes after THAM administration, plasma salicylate levels had dropped from 33 to 17 mg/100 ml. The highest blood pH was 7.57, CO_2 rose from 23.6 to 32.5 mM/L, HCO_3– from 22 to 31 mM/L and pCO_2 from 32.5 to 36.6 mm Hg. Na+, K+ and Cl– in plasma fell slightly but consistently.

Discussion. When THAM is used in treatment of experimentally produced acute salicylate intoxication, a significant increase in salicyluria is observed. Alkalinisation of the urine which results from this therapy may be the cause of this increase in salicylate excretion(4,5,6,7). Kaplan *et al.*(4) suggested that it might have been caused by HCO_3– and salicylate ions competing for absorption by the renal tubules. After finding that urine virtually free from HCO_3– had a higher level of salicylate when its pH was more alkaline, Kaplan also suggested that urinary pH at least partly determines rate of excretion of salicylate. Gutman *et al.*(5) and Schachter *et al.*(6) found that in man salicylate can be filtered at the glomerulus and reabsorbed and secreted by the renal tubules. Probenecid, which inhibits tubular activity, reduced the clearance of salicylate independently of urine pH, probably by inhibition of active tubular transport. When urine pH was below 6.0, there were indications of glomerular filtration and tubular reabsorption of salicylates. When urine pH was greater than 7.4 ratio of the clearance of salicylate to glomerular filtration rate (CFrS/CF) was greater than 1.0. When urine pH was kept above 7.50 by administering $NaHCO_3$ and probenecid simultaneously, a decrease in clearance of salicylate was found, supporting the belief that salicylate is actively secreted by the tubules. Weiner, Washington and Mudge(7) assessing

TABLE II. Salicylate, Electrolyte Conc., pH, pCO_2 and CO_2 Content of Plasma in 4 Dogs after Salicylate Admin. (100 mg/kg) and Treatment with THAM; Blood Samples Were Taken in Middle of Each Period. First figure in each column is avg of all measurements. Figures in parentheses indicate range.

Period	Time, min.	Salicyl, mg/100 ml	Na^+	K^+	Cl^-	HCO_3^-, mM/l	pH	Content CO_2, mM/l	pCO_2, mm Hg
			meq/l						
1. Control		0	153 (148–155)	3.2 (2.4–3.8)	114	19.9 (19.1–21)	7.34 (7.28–7.37)	21.0 (20.1–22.1)	37.8 (37–42.6)
2. Salicyl → inj.	0								
2.	15	30 (25–37)	151	3.5	—	22.1	7.27	23.6	49.7
3. THAM infusion	30	33 (22–54)	150 (145–154)	3.5 (3.2–3.7)	113	20.9 (18.1–27.2)	7.42 (7.37–7.57)	21.9 (19.0–28.1)	32.6 (30.6–34.9)
4.	45	29 (19–47)	140 (134–146)	3.6 (3.4–3.8)	103	27.6 (24.3–34)	7.52 (7.50–7.54)	28.9 (25.2–35.3)	34.8 (31.1–41.1)
5.	60	22 (16–28)	137 (132–142)	3.4 (3.1–3.8)	101	31.4 (27.8–35.7)	7.57 (7.51–7.67)	32.5 (28.9–36.9)	35.3 (26.8–40.2)
6.	90	19 (16–24)	146 (145–147)	2.8 (2.7–3.0)	102	28.0 (23.3–30.6)	7.51 (7.41–7.64)	29.1 (24.4–31.9)	36.7 (28.9–43.2)
7.	120	17 (10–23)	144 (141–150)	2.7 (2.6–3.0)	106	26.0 (21.4–28.9)	7.50 (7.45–7.53)	27.4 (22.3–30.1)	35.0 (31.3–39.8)

the relative roles of urinary pH and urine volume, found that a CFrS/CF ratio significantly greater than 1.0 with a urine pH more acid than that of blood could be produced when the ratio of volume of urine to glomerular filtration rate $\left(\frac{V}{GFR}\right)$ was .09. These authors concluded that salicylate was secreted by the tubule after observing that salicylate elimination in the urine was depressed by other substances like PAH and acetazolamide, which are also secreted by the tubules, and by compounds like probenecid that inhibit other tubular transport mechanisms. It was postulated that a passive ion trapping mechanism would cause reabsorption of salicylic acid.

Regardless of the final proof of the mechanism by which salicylate is reabsorbed or secreted, the present experiments indicate that both an alkaline urine and an increase in urine formation enhance elimination of salicylate by the kidney. For instance, in Exp. 4 the highest salicylate urinary excretion (4824 μg/min) was accompanied by the largest diuresis (10.2 ml/min). The marked salicylate output was attained although urinary pH (7.67) was not as high as the pH reported by others(4,7). Furthermore, protein binding of salicylate may be pH dependent: the lower the pH, the greater the binding. It is therefore important to have an alkalinization of body fluids so that this binding be minimal (personal communication). THAM, by producing an alkalosis of intra- as well as extra-cellular fluid, will therefore limit protein binding of salicylate and favor its renal excretion. For the same reason, acetazolamide, which produces a metabolic acidosis, may not be the best agent to combat salicylate intoxication, although acetazolamide induces excretion of an alkaline urine and has been advocated for treatment of salicylate poisoning(14,15). When THAM was first employed in treatment of experimentally produced metabolic and respiratory acidosis and in man, its possible usefulness in acute salicylate poisoning was apparent. The results of these experiments confirm this hypothesis. Since THAM has been found to be safe in humans(3,16,17) in doses similar to those used in the present experiments, it should be beneficial in treatment of infants and children suffering from salicylate poisoning. THAM has already been used in one such instance(18).

Summary. The effectiveness of tris (hydroxymethyl) aminomethane (THAM) in increasing salicyluria after intravenous injection of sodium salicylate was tested in experiments on 4 dogs. A marked drop of salicylate in plasma and increase in its excretion in urine was demonstrated. These ex-

periments also indicate that maximum excretion of salicylate by the kidney will occur when an elevation of body fluid pH and urine pH are accompanied by a marked diuresis. THAM, which promotes these changes, may be useful in treatment of salicylate poisoning in man.

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Received June 24, 1960. P.S.E.B.M., 1960, v105.

Effect of Internally Deposited Radioisotopes upon Blood Vessels of Cortical Bones.* (26108)

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Recent reviews by investigators dealing with internally deposited radioisotopes in bone state there is no evidence that injury to the bone was secondary to injury to small blood vessels directly supplying the bone(1, 2). They conclude that injury to the various cellular constituents of the bone appears to be a direct effect of the radiation upon them. This conclusion is based on the study of bones from radium patients in which activity is found throughout bone, and the alpha particles appear to be killing osteocytes directly.

In the present study we demonstrate that much of the damage in cortical bone is due to vascular occlusion. These studies have utilized dogs containing radium, plutonium or radiothorium in which a new technic of vascular injection of India ink-gelatin has been used to demonstrate the disturbance in blood supply. Unlike radium, which is distributed throughout bone, plutonium and radiothorium are localized on bone surfaces and the short range of the alpha particles located about haversian canals allows the major portion of the haversian systems and all the interstitial lamellae to be free of activity. Thus, if massive devitalization of bone is observed, it cannot be related to direct killing by alpha rays.

Materials and methods. The materials used were the mid-diaphyses of tibiae from 4 normal and 23 beagles that died or were sacrificed from 1 to 1724 days following administration of radium-226, plutonium-239 and

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* Supported by USAEC.

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TABLE I. Summary of Experimental Dogs.

Parent isotopes	Dose (μc/kg)	No. of animals
Plutonium-239	3.0	18
Radium-226	10.0	3
Radiothorium-228	.9	3
Control	—	6

radiothorium-228 (Table I). Seven of these animals were part of the Utah Beagle chronic toxicity studies of bone-seeking radioelements at the Radiobiology Laboratory, Univ. of Utah College of Medicine. Augmenting the chronic toxicity dogs were 15 plutonium dogs and 1 radium dog which were serially sacrificed to give information regarding distribution of the radioelements and sequence of histopathologic changes.

Young adult Beagles of mixed sexes between 14 and 24 months were given a single intravenous injection of one of the principally alpha emitting bone-seeking radioisotopes (plutonium, radium or radiothorium). Injection solutions were prepared in citric acid-sodium citrate buffer, 0.08 M in total citrate and pH 3.5(3). The chronic toxicity dogs were permitted to live until moribund. All dogs were anesthetized with sodium pentothal, each given 100 mg of heparin intravenously, and 15 minutes later, exsanguinated. A mixture consisting of Higgin's India ink, 1 part, and 14% solution of calfskin gelatin, 1 part, was injected (at 37°C) into the femoral artery of a disarticulated hindlimb. After injection of 400 ml of fluid, the limb was chilled (–20°C) to solidify the gelatin. The bones were then disarticulated, defleshed and radiographed. For this study adjacent 2 cm long specimens of the mid-diaphysis of the tibia and whole metatarsi were sawed and fixed in Zenker-formol and absolute acetone. Specimens fixed in Zenker-formol were embedded in celloidin, sectioned and stained with hematoxylin and eosin to demonstrate ink filled vessels and viable osteocytes (Fig. 4). Details of vascular injection as well as celloidin technics have been reported(4).

Specimens fixed in absolute acetone were used in preparing 50-80 μ thick transverse ground sections. The bone was first sawed to 200 μ in thickness with a special rotary saw. The sawed sections were then ground to the desired thickness between 2 ground glass plates using levigated alumina suspended in absolute alcohol. Water was avoided to insure against leaching of the isotopes. These sections were microradiographed on Lippmann film‡ with 10-Kv x-rays. Absorption of the x-rays in the section was such as to demonstrate different degrees of mineralization. Following the microradiograph, an autoradiogram of each section was obtained by exposing the sections on Eastman Kodak's NTB, 10 μ thick, "t" coat nuclear track plate for 30 to 50 days. The plates were developed in Kodak's D-19 for 4 minutes, fixed in acid fixer for 10 minutes, rinsed in tap water for one hour and allowed to dry in air.

Results. Autoradiographic findings. Two contact autoradiograms of a region of a transverse section of tibiae containing either plutonium or radium are presented to exemplify representative localization patterns. When plutonium or radiothorium was taken up by cortical bone, radioactivity was deposited on bone surfaces in varying concentrations depending on location. There was an intense concentration on the endosteal surfaces, a moderate concentration on periosteal surfaces, and on surfaces of resorption cavities and vascular channels, and a much lower concentration about haversian canals (Fig. 1). It should be emphasized that the range of the alpha particles of plutonium and radiothorium have short range in bone (about 24 μ), which meant that only a portion of the osteone about the canal is irradiated directly by alpha particles, while the remainder of the osteone and the surrounding interstitial lamellae were free of activity.

As the result of internal reconstruction, some of the initial deposition of plutonium or radiothorium on bone surfaces are absorbed into the blood stream. Subsequently, some of the plutonium or radiothorium then is redeposited as a lighter, diffuse pattern in the new generations of osteones.

When radium was taken up by cortical bone, the activity deposited throughout bone

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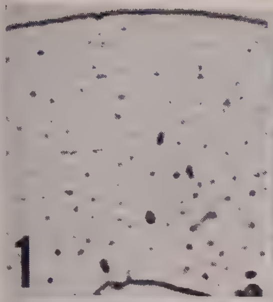
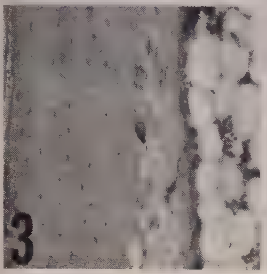
‡ Obtained from Gavaert Co. of America, Inc., Los Angeles, Calif.





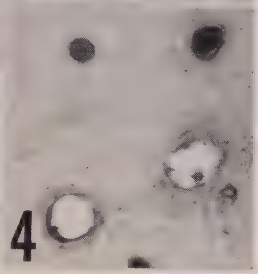


FIG. 1. A contact autoradiogram of a portion of transverse section of tibial diaphysis to show localization of plutonium on bone surfaces (endosteal, periosteal, haversian canals and vascular channels). Note large fraction of bone free of radioactivity (× 13).

FIG. 2. A contact autoradiogram of cortical bone containing radium to show intense deposits of radioactivity ("hotspots"), low "diffuse" deposition throughout remainder of bone and many punctate areas of moderate radioactivity (× 13).

FIG. 3. Endosteal surface of radius containing plutonium showing active bone resorption under the intense fibrosis. Celloidin section, H & E (× 68).

FIG. 4. A cross section of metatarsus containing plutonium and inj. with India ink-gelatin at autopsy to show osteones with India ink in haversian canal and viable osteocytes (upper right) and osteones with plugged canals and empty lacunae (upper left). Note much of the interstitial lamellae are with empty lacunae. Celloidin section, H & E (× 68).

both in areas of bone growth and in bone existing at time of administration of radium. The regions of bone growth were characterized by intense deposits of radium commonly called "hotspots," while the remainder of the bone exhibited a much less intense deposit called "diffuse" activity (Fig. 2). Also many punctate regions of moderate deposition were found about haversian canals.

Sequence of histopathologic changes in cortical bone containing plutonium. The earliest changes occurred at the endosteal surfaces of the compacta where initially there was a heavy concentration of plutonium. This resulted in destruction of endosteal cells within one week, followed by loss of osteocytes located within the range of the alpha particles deposited on the endosteal surfaces. In turn, this was followed by formation of a dense connective tissue (fibrosis) at 2 months and occurrence of abnormal bone resorption beneath the fibrosis at 3 months (Fig. 3).

Later changes observed in the bulk of the cortical bone, which contained principally a light concentration of plutonium about haversian canals, involved first the plugging of many haversian canals at 400 days post injection. The proof that the canals were functionally obstructed and not simply artifacts was supplied by sections of tibiae and metatarsi injected with India ink-gelatin. Fig. 12 exemplified a case of a dog containing radiothorium which showed a plugged vessel in a longitudinal section of metatarsus, and only India ink was found pooled on the periosteal surface and in the marrow cavity, in contrast to rich network of vessels filled with India ink in bones of control animals (Fig. 11). To date, the only observations of the character of the haversian canal plugs were that the majority have slightly basophilic staining properties and were not calcified, while a few plugs were of hypermineralized material.

Although approximately half of the canals were plugged at 400 days, the majority of the osteocytes appeared histologically normal, while only a few appeared shrunken. However, increasing burden time resulted in increased plugging of canals which in turn limited fluid circulation sufficiently to result in the death of osteocytes in affected osteones and adjacent interstitial lamellae. It should be emphasized again that the majority of these dead osteocytes were in regions free of activity. All the osteocystes within osteones having a blood supply were viable. The direct relationship of blood supply (India ink filled vessels) and viable osteocyte was apparent at 777 days after injection of plutonium (Fig. 4).

The fact that not all haversian canals were plugged allowed some internal reconstruction to occur; thus, new osteones were formed. However, the process of reconstruction at 2½ years in these animals was abnormal, re-

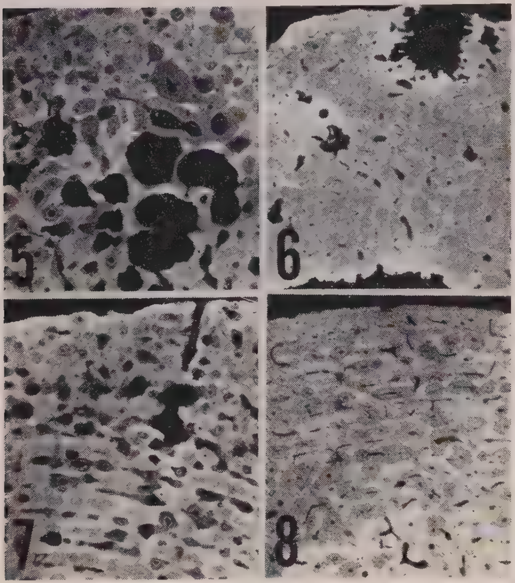


FIG. 5-8. Microradiograms of cross section of the tibial diaphysis (× 13).

FIG. 5. Compacta of dog given 10 μc/kg of radium. Note large resorption cavities, hypocalcified osteones and hypercalcified haversian canal plugs. High calcification appears white on the photograph.

FIG. 6. Compacta of dog given 0.9 μc/kg of radiothorium. Note large area of periosteal resorption, ragged endosteal surface, few small cavities and bland mineralization.

FIG. 7. Compacta of dog given 2.5 μc/kg of plutonium. Note occasional large cavities, numerous hypocalcified osteones and a few hypercalcified plugs.

FIG. 8. Compacta of control dog. Note that osteones are slightly less mineralized than interstitial lamellae.

sulting in formation of many large resorption cavities and abnormal osteones. In general, resorption cavities were slightly larger than average, irregular in shape and often filled with fibrous tissue. A few cavities were 4 to 6 times the size of a normal resorption cavity (Fig. 7 and 10). The abnormal osteones were generally structurally disorganized and abnormally calcified (Fig. 5, 7, 9 and 10).

No spontaneous fractures occurred in long bones of dogs given 2.5 μc/kg of plutonium in contrast to the numerous incidence of fractures in long bones of dogs given 10 μc/kg of radium and 0.9 μc/kg of radiothorium. Nevertheless, 2 animals given 0.9 and 0.27 μc/kg of plutonium developed microscopic osteogenic sarcoma arising from cortical bone.

Changes in cortical bones containing radium. Although only a few serial sacrificed dogs were studied, the compacta in animals given radium demonstrated similar changes. Besides the varieties of abnormal osteones and regions of necrotic bone observed in dogs given plutonium, the bones with radium contained unusually large cavities (Fig. 5). The portion of the compacta that was necrotic (loss of osteocytes) represented approximately 60-85% of the bone. These bones also differed in that numerous spontaneous fractures of the long bones were observed in animals given 10 μc/kg of radium.

Changes in cortical bone containing radiothorium. In contrast to radium and plutonium, concentration of activity about haversian canals in animals injected with 0.9 μc/kg of radiothorium resulted in the almost

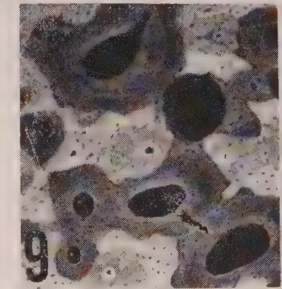


FIG. 9. Microradiograph of a cluster of hypocalcified osteones with enlarged haversian canals (× 68).

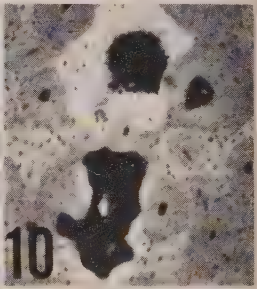


FIG. 10. Microradiograph showing an abnormally high mineralized osteone (white structure) and an adjacent irregular shaped resorption cavity (× 68).

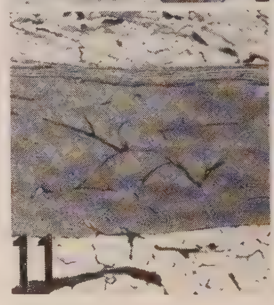


FIG. 11. Longitudinal section of a metatarsus inj. with India ink from a control dog. Note network of vessels filled with ink in the compacta, marrow and juxtaperiosteally. Celloidin section, H & E (× 13).

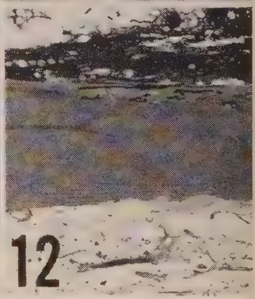


FIG. 12. Longitudinal section of a metatarsus inj. with ink from a dog given radiothorium. Note the single plugged vessel in compacta without ink, endosteal fibrosis, meager supply of ink filled vessels in the marrow and pooled ink on the periosteal surface. Celloidin section, H & E (× 13).

complete shut down of circulation and total necrosis of the compacta. This was best exemplified in the metatarsus whose vascular tree was injected with India ink in which the compacta was free of vessels containing India ink. The ink was located in meager amounts in the marrow and pooled on the periosteal surface. The region of pooled ink corresponded to the region of resorbed bone (Fig. 6 and 12). The few irregular resorption cavities shown in the microradiograph in Fig. 6 were formed early after the acquisition of radiothorium before the loss of total vascularity resulting in total inhibition of internal reconstruction. The bones illustrated in Fig. 6 and 12 were pre-injection bones, which had subsequently lost their viability (dead bone) as the result of loss of vascularity. The staining characteristics of non-viable bones were very poor and appeared quite uniform in mineralization in that osteones were only slightly less mineralized than interstitial lamellae (Fig. 6) as compared to the normal (Fig. 8).

Another constant finding was the occurrence of spontaneous fractures of long bones in every dog given 0.9 μc/kg of radiothorium.

Discussion. Injury to blood vessels has been regarded as a feature of irradiation lesions by many investigators who have given external irradiation, radium and polonium to animals and patients(5,6,7). However, there is no direct evidence that this is a major factor in causing damage to bone. Rowland, *et al.*(8) demonstrated plugged haversian canals and stated that this "implies the existence of widespread circulatory damage throughout bone." Our study shows by direct observation and a vascular injection technic that there is extensive functional vascular obstruction which results in bone necrosis.

The nature of the thrombosed haversian canals differs considerably from the changes in vascular tissue in other organs receiving irradiation(5,6,7). We have observed the apposition of mineralized matrix or secretion of a "matrix-like" material by the cells in the haversian canals. This may be simply the elaboration of mucopolysaccharide by vascular endothelium as shown by Curran(9) or of polysaccharide material observed in blood vessels after acute irradiation by Andersen (10). In our laboratory, plugging of haversian canals also has been observed to occur in the normal aging process in dogs.

The abnormal character of the osteones and the existence of large resorption cavities have been described in earlier reports dealing with radium patients(8,11,12,14). The abnormal calcification in form of hypercalcified rings has been seen in bones of older people (13) and Beagles. However, the character and scattered distribution of the large cavities in cortical bone induced by alpha emitters differs from the cavities seen in osteoporotic humans.

It is generally accepted that osteocytes can revert back into osteoblasts and function to lay down new bone. However, in the compacta of these animals much of the bone lacks osteocytes, which would mean that the blood vessels must bring in fibroblasts into the area to form osteoclasts and osteoblasts to participate in bone remodelling. If the aberrant cell is the fibroblast then it would be very difficult to relate this cell with direct alpha ray damage due to the fact that nearly all activity is located in the existing bone. The peculiar action of the osteoblasts could be the result of a disturbed local environment such as altered vascular supply. Nevertheless, direct injury by alpha rays must not be overlooked in that there is always a small amount of activity circulating in blood.

We note that the large cavities in cortical bone containing radium contained either fibrous connective tissue or bone marrow cells. It is believed that one of the factors concerned with formation of large cavities is increased resorption due to enhanced devitalization of bone at areas of intense irradiation ("hotspots"). This is supported by observation in which we relate devitalized region of bone to "hotspots" in high resolution autoradiography studies in our laboratory. In the region of "diffuse" deposits of radium the osteocytes are viable. Often the intensity of the irradiation at "hotspots" is too damaging to allow bone remodelling to be carried out. It is logical to speculate that the continual presence of the low intensity "diffuse" deposits of radium in bone may be sufficiently injurious to inhibit the apposition of bone, but allow for regeneration of circulating bone marrow cells and fibrous connective tissue.

Occurrence of spontaneous fractures in long bones of dogs given 10 μc/kg of radium and 0.9 μc/kg of radiothorium should be considered. Spontaneous fractures are quite common in radium patients(1,11,14). In our study it is believed that the events which weaken the bones containing radiothorium are principally due to total devitalization and increased mineralization of cortical bone aided slightly by resorption of endosteal and periosteal surfaces. In the cases of bones containing radium, the weakening can be attributed partially to devitalization of bone and partially to the large cavities (diffuse osteoporosis). Of course, the adverse effects of alpha emitters on collagen and bone matrix, whatever they may be, must not be overlooked.

Summary. It has been observed that after an extensive latent period following acquisition of plutonium, radiothorium and radium in cortical bone, plugging of the haversian canals occurs which in turn results in bone necrosis. Investigation of this phenomenon with a vascular injection technic and a bone-seeking alpha emitter (plutonium) which deposits on surfaces of haversian canals and allows the remainder of the osteones and adjacent interstitial lamellae to be free of activity, show that death of osteocytes is principally the result of disrupted blood supply rather than direct alpha ray killing. With radiothorium, concentration of alpha particles about haversian canals was sufficient to result in total plugging of the vascular tree within the haversian canals resulting in complete bone necrosis and inhibition of internal reconstruction of cortical bone. With radium and plutonium only about half of the canals are plugged, which allows internal reconstruction to proceed; however, the nature of the remodelling in this environment is abnormal (formation of large cavities and abnormal osteones). It is also suggested that both devitalization of bone and occurrence of large cavities weakens the bone which in turn results in spontaneous fracturing of long bones.

The authors are indebted to the Director of the Radiobiology Lab., Prof. T. F. Dougherty, whose cooperation facilitated this work. Technical assistance of Milton Lowe and Renon Mical is particularly appreciated.

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Received July 22, 1960. P.S.E.B.M., 1960, v105.

Agar Gel Diffusion Studies with Sera and Urinary Proteose from Patients with Certain Renal Diseases.* (26109)

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(Introduced by S. Howard Armstrong, Jr.)
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Isolation and characterization of a proteose in urines of patients with certain active renal diseases has been reported(1). We are investigating whether this carbohydrate-rich proteose might have antigenic properties and be implicated in an autoimmune process in renal disease. The demonstration of reactions by the agar diffusion technics between sera and urinary proteose from patients with certain renal disease is reported here.

Methods and procedures. Urinary proteose was prepared according to the method previously reported(1). The proteose containing filtrate was brought by boiling to half the volume of the original urine, and served as antigen.† Double diffusion agar studies were carried out by the Korngold modification of the Ouchterlony technic(2) and with a modification of the Oakley-Fulthorpe technic(3). The modification was addition of 3.75 g % of ammonia-free glycine to the 2 concentrations of agar employed. Difco Noble Agar was used and no preservative added. Two ml of proteose solution were added to 5.0 ml of antigen agar and the serum layered over the clear zone agar. The tubes were sealed with modeling clay and incubated at 27°C for periods up to 30 days. Patients from the Nephritic Research Clinic of Hektoen Inst. for Medical Research who had been followed for from 6 months to 3 years and who had been diagnosed clinically and with renal biopsies were selected for this preliminary study.

Results. One hundred and eighty-two tests were performed with 44 sera and 43 urinary proteose preparations. Thirteen sera and 14 urinary proteose reacted. The type of reaction observed is illustrated in Fig. 1 and relationship of patient diagnosis to reaction is summarized in Table I. The diagnosis of glomerulonephritis was established by clinical evaluation and renal biopsy, while diagnoses of Kimmelstiel-Wilson disease, pyelonephritis and nephrosclerosis rest on clinical evidence and the usual laboratory findings. Only the predominant pathologic diagnosis is listed; many of the biopsies also revealed concomitant interstitial nephritis. One patient with chronic glomerulonephritis and systemic lupus erythematosus and on steroid therapy failed to react in autologous system but did cross react with the proteose and serum of a patient with active glomerulonephritis. The urinary proteose of a patient with biopsy evidence of chronic membranous glomerulonephritis and also on steroid therapy cross reacted with the serum of a patient with biopsy evidence of chronic glomerulonephritis. The sera and urinary proteose of a third patient on steroid therapy at time of study and with renal biopsy evidence of chronic glomerulonephritis and acute focal interstitial nephritis, failed to react in autologous or cross systems.

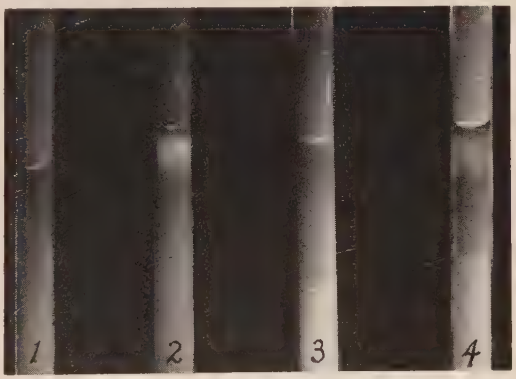

FIG. 1.

1. T.G.*/R.McK.* (neg)
2. T.G./E.S.† (pos)
3. E.R.*/J.L.‡ (neg)
4. E.R./E.R. (pos)

* Chronic glomerulonephritis.
† " " on steroid therapy.
‡ Nephrosclerosis.

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* Supported by grant from Nat. Inst. Health.

† Solubilization of both albumins and globulins on boiling with proteose between pH 5 and 6 has recently been observed; antigen thus prepared cannot be considered free from other proteins.

TABLE I. Relationship of Diagnosis to Reactions.

Sera	Urinary proteose	Positive reactions
Glomerulonephritis	Glomerulonephritis	21
"	Glomerulonephritis + systemic lupus erythematosus	1
Glomerulonephritis + systemic lupus erythematosus	Glomerulonephritis	1
Kimmelstiel-Wilson + pyelonephritis	"	6
Idem	Kimmelstiel-Wilson + pyelonephritis	1
"	Nephrosclerosis*	1
Nephrosclerosis*	Kimmelstiel-Wilson + pyelonephritis	1
"	Glomerulonephritis	3
"	Nephrosclerosis*	1

* This patient also had parathyroid adenoma, positive urine culture at one time, and urinary sediment contained red blood cell and white blood cell casts. No renal biopsy is available.

Attempts to obtain reactions between orosomucoid‡ and positive and negative sera failed. Seven positive and 11 negative sera did not react with orosomucoid (100 mg % in phosphate buffer at pH 6.9) either before or after boiling. Tests between 2 normal sera, 2 sera from patients with pneumonia and 12 positive reacting urinary proteose were negative. Four patients with nephrosclerosis also failed to react.

The clinical and laboratory findings listed for each patient were those present on the day blood was drawn and urinary proteose or urine treated as for proteose was prepared. Table II illustrates occurrence of various factors in reactors and non-reactors. Proteinuria and significant urinary infection were more frequent in positive reacting patients than in non-reactors and apparently the sera and urinary proteose from patients with the nephrotic syndrome gave highest evidence of reaction and interreaction.

Discussion. In those patients with glomerulonephritis and pyelonephritis, it is interesting to speculate whether or not the cross reactions are due to the antigenicity of the protein derivatives from the interstitial component of the disease. The findings of Goodman, Greenspan and Krakower(4) that in the dog, renal tubular epithelial cells possess antigens in common with the epithelial cells of the renal glomerulus, and of Baxter and Goodman(5) that the renal medulla is likewise capable of producing a nephrotoxic antiserum, tend to support this view. Failure of the 3 patients on steroid therapy to react in autologous system while 2 did cross react raises the question whether steroid therapy prevents or attenuates the autoimmune mechanism as measured by these methods. Liu and McCrory(6), using the Ouchterlony double diffusion agar technic, tested sera from patients with glomerulonephritis and the nephrotic syndrome against whole extracts of human kidney, liver and lung. They reported an overall higher incidence of reactors in patients with the nephrotic syndrome. We have found a higher percentage of reactions with sera and urinary proteose from patients with the nephrotic syndrome. The reported reactions of sera and proteose in man as evidenced by double diffusion agar technics have their counterparts in the work of Witebsky, Rose and Shulman(7), suggesting the possibility of an autoimmune process much the same as the abnormal thyroglobulin in thyroiditis.

TABLE II. Occurrence of Factors in Reactors and Non-reactors.

		Symptoms	Abnormal urinary protein	Abnormal urinary red blood cells	Antistreptolysin-O titers			Urinary infection		Nephrotic syndrome
					Normal	Elevated	Unknown	+	?	
		%								
Sera	+	48	81	71	72	23	5	42	5	33
	0	30	72	62	69	23	8	31	4	20
Urinary proteose	+	33	100	75	61	33	6	44	3	58
	0	27	70	63	61	30	9	26	3	29

+ = Reactors. 0 = Non-reactors.

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‡ Kindly supplied by Dr. Richard J. Winzler.

Summary. Visible precipitin bands have been demonstrated by double diffusion in agar studies with human urinary proteose and nephritic sera. These reactions were observed in autologous and cross systems. It appears that sera and urinary proteose obtained from patients with the nephrotic syndrome are the most likely to react.

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Received July 26, 1960. P.S.E.B.M., 1960, v105.

Effect of *Sterculia Foetida* Oil on Uptake of Water by the Avian Egg Yolk.*†
(26110)

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Addition of sterculic acid(1,2), *Sterculia foetida* oil(1), malvalic acid(2), or cottonseed oil(3) to the diet of laying hens, causes pink discoloration in cold storage eggs. Schaible and Bandemer(4) showed that the pink color was imparted by a complex of iron with conalbumin. Evans, *et al.*(5) using paper electrophoresis reported that protein migrated from the white to the yolk during storage. This suggests changes in the egg which allow passage of iron and conalbumin across the vitelline membrane(4). The purpose of this investigation was to determine the effect of *Sterculia foetida* oil on the water passage into intact yolks *in vitro*.

Experimental. A preliminary experiment was designed to study the effect of "clutch size" on water uptake by the yolk. In Experiment I a group of 65 randomly selected S. C. White Leghorn hens were housed in individual wire cages and fed a practical laying diet *ad libitum.* Eggs were collected daily at 3:00 P.M. for 21 consecutive days and stored in the refrigerator at 7°C overnight. The next morning the eggs were tested for water uptake by the method of Orru(6). The intact yolk was placed in a tared beaker and weighed. Deionized water was added and the contents incubated at 37.0 ± 1.0°C for 3 hours. Following incubation the liquid was removed from the beaker with the aid of an aspirator and blotted with filter paper. The beaker and its contents were weighed and the increase in weight was considered to be water uptake.

On the basis of the clutch size data (Table I), hens from Exp. I averaging 2, 3, and 4 day clutches were divided randomly into 4 lots of 8 or 9 birds each for Exp. II. Housing, laying diet, collection of eggs, and method for determination of water uptake by egg yolks were similar to procedures employed in Exp. I with the following exceptions: Gelatin capsules containing supplements of *Sterculia foetida* oil‡ or corn oil§ were administered daily. Hens from Lots 1 and 2 received a supplement of 0.30 g of corn oil daily, while Lots 3 and 4 received a mixture of 0.15 g *Sterculia foetida* oil and 0.15 g corn oil. The

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* Supported in part by a grant from Mead Johnson & Co., Evansville, Ind.

† Preliminary report presented in *Fed. Proc.*, 1960, v19, 221.

‡ Extracted from the whole ground nut of the Java Olive tree, *Sterculia foetida*.

§ Mazola Oil, Corn Products Co., New York.

TABLE I. Influence of Clutch Size on Water Uptake of Egg Yolks and on Yolk Weights.

Size of clutch, eggs	No. of hens	Avg water uptake of yolks, g*	Avg yolk wt, g*
1	8	1.41 ± .28^{a}† (50)‡	19.84 ± 1.54^{a}† (53)‡
2	16	1.06 ± .26^{b} (129)	18.68 ± 1.26^{b} (144)
3	18	.99 ± .23^{b} (190)	18.73 ± 1.02^{b} (218)
4	8	.92 ± .15^{b} (86)	18.44 ± .96^{b} (96)

* Means with different superscripts are significantly different $(P < .01)$.
† Mean ± stand. dev.
‡ No. of yolks tested.

eggs were stored at 7°C overnight (Lots 1 and 3) or for one month (Lots 2 and 4). In Lots 2 and 4 incubation time was reduced from 3 to 2 hours because many of the yolks ruptured during the 3 hour incubation period. The Halphen test(7) was used to determine presence of *Sterculia foetida* oil in egg yolks. Measurement of water uptake by intact yolks was begun when egg yolks from Lots 3 and 4 gave a positive Halphen test.

Results. The influence of average clutch size on water uptake of egg yolks from hens fed a practical laying diet (Exp. I) is shown in Table I. Water uptake data on yolks obtained from hens with an average of more than 4 eggs per clutch were not included because of the small number. Average water uptake of yolks from hens that had an average clutch size of one egg was significantly higher $(P<0.01)$ than those from hens with a clutch size of 2, 3, or 4 eggs(9). No significant differences in water uptake were observed among yolks from the 2, 3, or 4 egg clutches.

After 6 days of daily administration of oil supplements, all the yolks tested from hens receiving *Sterculia foetida* oil (Exp. II, Lots 3 and 4) gave a positive Halphen test, whereas yolks from hens receiving corn oil (Lots 1 and 2) gave a negative test. The positive Halphen test indicated presence of sterculic acid(8) or a closely related compound in the yolk. Average water uptake of yolks and yolk weights of eggs from hens receiving corn oil or *Sterculia foetida* oil supplements is shown in Table II. After storage overnight, differences in water uptake and in yolk weights between eggs from corn oil and *Sterculia foetida* oil fed hens (Lots 1 and 3) were not significant. After storage of one month, significantly greater water uptake $(P<0.001)$ was found in the eggs from the *Sterculia foetida* oil fed group (Lot 4). Yolks of eggs from the *Sterculia foetida* oil group (Lot 4) were 20% heavier and took up 80% more water than those from the corn oil group (Lot 2).

Discussion. Differences in water uptake between clutch sizes may be influenced by differences in yolk weights (Table I) because yolk weights of clutch size 1 were significantly greater than sizes 2, 3, or 4 $(P< 0.01)$. Orru(6) reported higher water uptake values than those obtained in Exp. I. Orru(10) also reported that water uptake increased with storage time of the egg previous to incubation. Van Tienhoven, *et al.*(11) reported that average water uptake of fresh yolks was 1.268 g, but clutch size was not given. In our experience using Orru's(6) method of water uptake, the one egg clutch should be differentiated from the 2-4 egg clutch.

Increased water uptake by yolks from eggs in the *Sterculia foetida* oil lot occurs only after storage longer than overnight. Physical or chemical changes probably take place during storage of *Sterculia foetida* oil eggs which

TABLE II. Influence of Storage on Water Uptake and Yolk Weight of Egg Yolks Produced by Hens Fed *S. foetida* Oil or Corn Oil.

Lot	Supplement	Duration of storage at 7°C	Avg water uptake of yolks, g	Avg yolk wt, g
1	Corn oil	Overnight	.95 ± .29*(77)†	18.61 ± 1.07*(95)†
2	" "	1 mo	6.13 ± 2.07 (69)	19.36 ± 1.36 (89)
3	*S. foetida* oil	Overnight	1.03 ± .09 (31)	19.24 ± .09 (46)
4	" "	1 mo	11.12 ± 4.64 (11)	23.35 ± 1.74 (28)

* Mean ± stand. dev. † No. of observations.

do not occur during cold storage of corn oil eggs. This is supported by the observation that the putty-like yolks previously reported by Shenstone and Vickery(2) were produced only during cold storage of eggs from the *Sterculia foetida* oil lots. If eggs were stored at room temperature, this did not occur. Pink discoloration of eggs occurred only in eggs from Lot 4.

Summary. The yolks of eggs from hens having a one-egg clutch average took up significantly more water than those from hens having 2-4 egg clutch sizes. Yolks from hens having 2, 3, and 4 egg clutch sizes showed no significant differences in water uptake. Yolks from eggs stored overnight and from hens given *Sterculia foetida* oil were not significantly heavier and did not take up more water than eggs from hens given corn oil. However, when the eggs were stored one month the yolks from the hens fed *Sterculia foetida* oil increased 20% in weight and showed an 80% greater water uptake than those from the corn oil fed hens.

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Received July 29, 1960. P.S.E.B.M., 1960, v105.

Relation of Age to Radiomagnesium Exchange in Bone.* (26111)

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Evidence suggests that the reactivity of the skeleton, as measured by isotope exchange, declines with age(1). Studies in our laboratory have shown that radiosodium exchange in young bone exceeds that of old bone in both the rat and cat(2). A direct relationship between liberation of magnesium from skeleton and rate of bone growth has been demonstrated by Duckworth and Godden(3). The observed depletion of bone magnesium in calves fed a magnesium deficient diet(4), together with the finding of a normal bone content in lactating cows with hypomagnesemia(5), also suggests that bone magnesium is more labile in young animals.

This report concerns exchange of radiomagnesium in the bones of rats of various ages. Data are also included on age changes in bone content of stable magnesium.

Methods. Magnesium^{28} (T½ 21 h.) obtained from Brookhaven National Laboratories, was supplied as a solution of $MgCl_2$ in concentrated HCl. Sodium hydroxide was added to bring the pH to about 5. Due to the short half-life of the isotope it was necessary to inject more than trace amounts of stable magnesium. The amount actually injected was in the range of 0.1 to 0.16 meq. Mg/100 g body weight, and average dose of Mg^{28} was 4×10^5 c/m/100 g body weight.

Groups of female rats aged 20, 30, 60, 180 days were injected with measured amounts of the isotope intraperitoneally and after 4 hours were exsanguinated by decapitation. In some of the 180-day and 30-day animals,

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* Supported by grants from U. S. Atomic Energy Comm., Contract with University of Rochester, and Nat. Inst. of Arthritis and Metab. Dis.

TABLE I. Experimental Results.

Age, days	Wt, g	No. of animals	Equilibration time, hr	Bone water, %	Bone magnesium, meq/g wet wt	% Mg^{28} exchanged
20	44- 48	7	4	39.1 ± 1.7*	.18 ± .02*	31 ± 4.2*
30	60-100	12	4	24.2 ± 3.3	.25 ± .02	40 ± 8.3
		8	14	26.6 ± 1.8	.25 ± .02	36 ± 7.5
60	130-180	4	4	17.9 (17.7-18.4)†	.29 (.28-.30)†	6.2 (2.1-7.6)†
180	170-225	10	4	12.7 ± 1.1	.35 ± .05	4.1 ± 1.9
		6	14	13.9 ± 3.8	.30 ± .02	5.2 ± 1.2

* Stand. dev. † Range.

the experimental period was extended to 14 hours. All of the animals were fasted during the experiment, except the 30-day rats which were sacrificed after 14 hours.

After sacrifice, the hind limbs were removed and the long bones stripped of muscle and periosteum. The cancellous bone was removed and the marrow separated from the remaining cortical bone, which was placed at once in a tared vial. Radioactivity of bone and serum was determined in a scintillation counter, the usual corrections being made for decay and resolving time.

After drying to constant weight in a vacuum oven at 65°C, the samples were ashed in a muffle furnace at 525°C for 48 hours. The ash was dissolved in HCl, filtered, and made up to volume.

Serum magnesium was determined by the method of Orange and Rhein(6). Prior to anlaysis of the bone ash solutions, calcium and phosphorus were removed by passage through a cation exchange column consisting of a 0.5 x 37 cm column of the sulfonate polystyrene resin, Dowex-50, 50-100 mesh. An amount of bone ash solution corresponding to 50-80 mg of dry bone was added to the column and allowed to absorb on the resin. This was then eluted with 0.7 N HCl at a flow rate of about 0.7 cc/min. The initial 25 cc was discarded as it contained the phosphorus, and the following 125-150 cc were collected, and analyzed for Mg by the method of Orange and Rhein. The column was regenerated with 100 cc of 2 N HCl, and washed with distilled water.

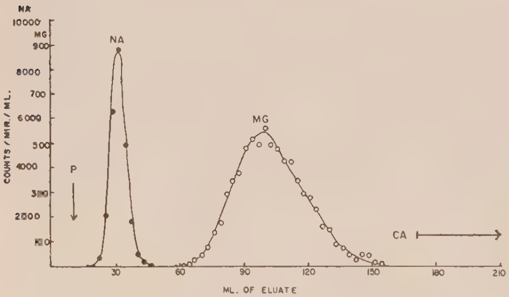

FIG. 1. Elution pattern of bone ash solution to which Na^{22} and Mg^{28} had been added.

Fig. 1 demonstrates separation of P, Na, Mg, and Ca in various portions of the eluate, utilizing the isotopes of Na and Mg. In a series of 12 runs, an average of 95% (s.e. 1.6) of the added Mg^{28} was recovered. The solutions used in these trials also contained 0.004-0.016 meq. Mg and about 0.5 meq. Ca, to simulate the proportion of these 2 ions as they occur in bone. It was found that addition of larger amounts of either Mg or Ca caused a shift in the elution pattern.

Results are summarized in Table I. It is apparent that the exchange of Mg^{28} is much (5-10 times) greater in the 20- and 30-day-old animals than in the 60- and 180-day-old groups. Per cent exchange is calculated as the ratio specific activity† bone/specific activity serum (x100). Prolonging equilibration period to 14 hours did not increase the exchange.

The Table also illustrates the progressive decline in bone water content and the progressive increase in stable magnesium content as the animals become older.

Discussion. The exchange of Mg^{28}, expressed as bone: serum specific activity, is much more rapid in the younger than in the older animals. This finding is in keeping with the age differences which we have previously reported for radio-sodium exchange in bone(7), though the latter isotope exchanges more rapidly at all ages than does Mg^{28}. Langemann(8) has reported that Mg^{28} accumulates in the bones of young rats about twice as fast as in the adult, when results were expressed as per cent of injected dose per gram of bone. This author also found that dietary Ca and Mg influenced uptake of Mg^{28} in bone. In this connection it should be noted that the 30-day-old animals in our experiments were fasted for 12 hours prior to injection of Mg^{28}, whereas the 20-day-old animals were allowed food during this period. The lowered uptake of Mg^{28} in the latter group may have been due in part to this necessary circumstance.

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† Counts/min./meq Mg.

Changes in bone water content are in keeping with those previously described in our laboratory(2). The increase in stable magnesium content is analagous to that previously noted for Na and Ca. It is apparent that experiments involving bone composition should be designed with this age factor in mind.

It is of interest that for about 10-15 minutes following injection many of our animals exhibited decreased activity and responsiveness, and stretching of the hind limbs. In a preliminary study of blood levels it was found that serum magnesium concentration rose following injection of the isotope solution to a value 50-100% above normal within 20-30 minutes, subsequently falling to normal in 4 hours. Simultaneous measurements of serum Mg^{28} confirmed the observation of Rogers and Mahan(9) that the level drops rapidly during the first 4 hours, followed by a much slower decline.

Neuman has shown that the magnesium of bone is a surface-limited ion(10). The greater degree of exchange exhibited by the bone of the young animal may be related to the smaller crystal size(11), or to the greater water content, implying a greater degree of accessibility to circulating body fluids(12).

The chromatographic method for separating Mg from Ca and P proved to be satisfactory. The method described here is essentially the same (though a small column was used) as that previously reported from our laboratory(13). It is essential that these interfering ions be removed, at least in bone, prior to determination of Mg by the method of Orange and Rhein. We found that failure to do so gave values which were about double those found by the chromatographic method for which it is known that recovery of added Mg^{28} is satisfactory. Others have also reported the use of Dowex columns for separation of Mg and Ca(14,15).

Summary. In the rat, the exchange of Mg^{28} in cortical bone occurs much more rapidly in young animals than in old. The stable magnesium content of bone increases with age, varies inversely with bone water content. A method for separating Mg from Ca and P by cation exchange chromatography is described.

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Received July 29, 1960. P.S.E.B.M., 1960, v105.

Effect of Tetrazolium Salts on Selected Bacterial Species.[*] (26112)

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The reduction of tetrazolium salts by living cells has been utilized extensively as an indicator of active metabolic processes(1-13). However, proper evaluation of the histochemical technic involving tetrazolium depends on understanding the effects of these salts *per se* upon living systems. Attempts have been made to characterize the effects on bacteria by studying their influence on growth and on isolated enzyme systems(15). The following communication deals with a detailed study of the growth inhibitory effects of these compounds on a number of bacterial species.

Method. *Escherichia coli, Salmonella typhimurium, Shigella sonnei, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes* and *Bacillus subtilis* cultures were isolated in our laboratory and carried on Trypticase Soy Agar (B. B. L.). Heart Infusion Broth (Difco), dispensed in 4 ml amounts in 15 x 125 ml tubes fitted with metal closures, was used in the experiments. The tetrazolium compounds used included 2 monotetrazolium salts, triphenyl tetrazolium chloride (TTC) and iodo-nitrotetrazolium (INT) and three ditetrazolium salts, neotetrazolium (NT), blue tetrazolium (BT) and nitro-blue tetrazolium (NBT).[†] Stock solutions of 0.5% were prepared in distilled water, autoclaved and diluted in broth to final concentrations of 2.0 to 500.0 μg/ml (10^{-6} to 10^{-2} moles/liter). Controls contained sterile distilled water instead of tetrazolium solutions. All experiments were carried out in duplicate. Each tube, inoculated with 0.05 ml of an 18 hour broth culture of the organism to be tested, was incubated at 37°C for 24 hours, at which time subcultures in tetrazolium-free media were made. The lowest concentration of each tetrazolium salt which completely prevented growth on subculture was considered the minimal lethal dose (MLD).

Results. MLD values for gram negative and gram positive organisms are shown in Tables I and II. The ditetrazolium salts (NT, BT, NBT) were more effective as bacterial growth inhibitors than the monotetrazolium salts (TTC, INT). This difference is even more striking when the molarity of the solutions is considered. The order of decreasing inhibitory action for both gram negative and gram positive organisms was generally NBT, NT, BT, INT, and TTC. Gram negative organisms were found to be more resistant than the gram positive organisms.

TABLE I. Minimal Lethal Doses of 5 Tetrazolium Compounds on Gram Negative Organisms (24 Hr, 37°C).

Tetrazolium salts	Organisms	Minimal lethal dose μg/ml	Minimal lethal dose Molarity
Monotetrazolium			
TTC	*E. coli*	>500	10^{-2} *
	Sal. typhimurium	>500	10^{-2} *
	P. vulgaris	>500	10^{-2} *
	Sh. sonnei	>500	10^{-2} *
INT	*E. coli*	>500	10^{-3} *
	Sal. typhimurium	>500	10^{-3} *
	P. vulgaris	>500	10^{-3} *
	Sh. sonnei	500	10^{-3}
Ditetrazolium			
NT	*E. coli*	125	10^{-4}
	Sal. typhimurium	250	10^{-4}
	P. vulgaris	500	10^{-3}
	Sh. sonnei	32	10^{-5}
BT	*E. coli*	250	10^{-4}
	Sal. typhimurium	500	10^{-3}
	P. vulgaris	>500	10^{-3} *
	Sh. sonnei	125	10^{-4}
NBT	*E. coli*	125	10^{-4}
	Sal. typhimurium	125	10^{-4}
	P. vulgaris	125	10^{-4}
	Sh. sonnei	8	10^{-5}

* Molarity of highest concentration tested; no inhibition observed.

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[*] This project has been aided by grant from Nat. Inst. Health, U.S.P.H.S.

[†] TTC, triphenyl tetrazolium chloride; INT, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride; NT, pp′-diphenylene-bis-2-(3,5 diphenyl tetrazolium chloride); NBT, 2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride; BT, 2,2′,5,5′-tetraphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene) ditetrazolium chloride.

TABLE II. Minimal Lethal Doses of 5 Tetrazolium Compounds on Gram Positive Organisms (24 Hr, 37°C).

		Minimal lethal dose	
Tetrazolium salts	Organisms	µg/ml	Molarity
Monotetrazolium			
TTC	*Staph. aureus*	>500	10^{-2} *
	Strep. pyogenes	32	10^{-3}
	B. subtilis	32	10^{-3}
INT	*Staph. aureus*	64	10^{-4}
	Strep. pyogenes	4	10^{-5}
	B. subtilis	16	10^{-5}
Ditetrazolium			
NT	*Staph. aureus*	8	10^{-5}
	Strep. pyogenes	<2	10^{-6}
	B. subtilis	<2	10^{-6}
BT	*Staph. aureus*	8	10^{-5}
	Strep. pyogenes	4	10^{-6}
	B. subtilis	4	10^{-6}
NBT	*Staph. aureus*	8	10^{-5}
	Strep. pyogenes	<2	10^{-6}
	B. subtilis	<2	10^{-6}

* Molarity of highest concentration tested; no inhibition observed.

With one exception all the salts tested were lethal to gram positive organisms in concentrations ranging from less than 2 up to 64 µg/ml. However, *Staph. aureus* was resistant to the action of TTC even at 500 µg/ml, the highest concentration used. In the case of the gram negative organisms tested, TTC was found ineffective, even in concentrations of 500 µg/ml, while the other salts showed varying degrees of antibacterial activity. *Sh. sonnei* was found to be most sensitive to the inhibitory effects of tetrazolium salts, and *P. vulgaris* the least sensitive.

Intense reduction of the tetrazolium salts to colored formazans was generally associated with a decreased lethal effect. This was more apparent in the case of the gram negative organisms, which were less affected by the tetrazolium salts, than in the case of the gram positive organisms.

Discussion. The previously reported(1) inverse relationship between inhibition of bacterial growth by the tetrazolium compounds and reduction of these compounds to their respective formazans was confirmed. It has also been shown that the tetrazolium compounds are more lethal to gram positive organisms than to gram negative organisms. These results confirm the previously reported effects of NT(1). In the living state the gram positive bacteria are generally more permeable to dyes. They are also more susceptible than gram negative organisms to the bacteriostatic effect of triphenylmethane dyes and to the action of surface tension reducing agents(14). These properties could account for the different reactivity to tetrazolium of the 2 bacterial groups, since tetrazolium salts are surface tension reducing agents and have certain structural resemblance to triphenylmethane dyes. Quantitative differences in the metabolism of the 2 groups of bacteria might also play a role.

The inverse relationship between tetrazolium reduction and growth inhibition reflects the necessity for active cellular metabolic processes in the reduction to formazan. Growth inhibition is probably due to interference with cellular enzyme systems, thus blocking pathways for continuous transference of electrons to the tetrazolium salt.

A similarity may be noted between the inhibitory effectiveness of the tetrazolium salts on bacterial growth reported here and their influence on pseudo - cholinesterase(15) While this enzyme is not commonly found in bacteria, tetrazolium salts may exert inhibitory effects on bacterial enzyme systems through a similar mechanism. The presence of the biologically reactive quaternary amine group in the tetrazolium molecule tends to support this contention. An earlier study (16) reporting higher toxicity for BT in mammals as compared with the monotetrazolium derivatives is in accord with findings in bacteria herein described.

Summary. The influence of 5 tetrazolium compounds on growth of various bacterial species is reported. Gram negative organisms are more resistant to the inhibitory effects of these compounds than are the gram positive organisms. The ditetrazolium salts are more lethal to the bacteria than are the monotetrazolium salts.

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Received August 1, 1960. P.S.E.B.M., 1960, v105.

Immunologically Competent Cells in Adult Mouse Liver: Studies with Parent-to-Hybrid Radiation Chimeras. (26113)

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Evidence has been accumulated that tissues other than spleen, lymph nodes, and bone marrow contain immunologically competent cells. Peripheral white blood cell fractions (1,2), thymus(3,4), and liver, kidney, and heart muscle(4) contain cells able to confer adoptive immunity to transplantation antigens and to cause rejection of heterologous or homologous bone marrow following implantation in X-irradiated mice.

To obtain further evidence of the immunologic potentialities of adult liver tissue, we grafted liver cells into sublethally irradiated F_1 hybrids, using inbred parental strain mice as donors. In this genetic situation, it is expected that only immunologically active cells of the grafted liver can react positively against the F_1 hybrid antigens. Therefore, a fatal reaction should occur in the F_1 recipients of certain strain combinations, similar to the wasting disease described after parental spleen(5,6), thymus(7), or leukocyte(8) implantation.

Methods. Sixteen- to 20-week-old male and female (C57BL × 101)F_1, (C3H × 101)F_1, and (101 × C3H)F_1 recipient mice were exposed to a single dose of total-body radiation of 600 or 900 r. Mice were irradiated in a revolving partitioned lucite cage under the following conditions: 300 kvp, 20 ma, 4.7 mm of Be inherent and 3 mm of Al added filtration, 100 cm target-to-mouse distance, hvl 0.45 mm of Cu, 76 r/min.

Within 1 hour after irradiation, each 600-r irradiated mouse received intraperitoneally a suspension of parental strain liver cells, equivalent to 0.25 of a whole liver in 0.5 to 1 ml of Tyrode's solution. Liver cell suspensions were prepared by chopping the organ with scissors and by repeated aspiration of small fragments through a syringe. The preparation was finally passed through a wire mesh screen, and penicillin was added.† Each 900-r irradiated mouse received intravenously within 1 hour, *via* the tail vein, 1 femur-equivalent (about 10×10^6 nucleated cells) of isologous bone marrow (IBM). Subsequently these mice were given an intraperitoneal inoculation of parental liver cells equivalent to 0.1 of a whole liver. Marrow

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

† The final suspension of liver cells contained in all instances a mixture of parenchymal, littoral, and biliary cells; these are referred to throughout the text as simply ''liver cells.''

TABLE I. Survival of 600-r Irradiated F_1 Hybrid Mice Injected with Parental Strain Liver Cells.

		90-day survival of recipients			
Recipients	Donors	No. treated	No. surviving	% surviving	Time of death (days after irrad.)
(C57BL × 101)F_1	Radiation control	30	30	100	
	(C57BL × 101)F_1	25	24	96	18
	C57BL	40	0	0	13–25
	101	40	0	0	12–17
(C57BL × 101)F_1 + IBM	C57BL	30	20	66	25–53
	101	40	32	80	19–56
(C3H × 101)F_1	Radiation control	20	19	95	17
	(C3H × 101)F_1	20	17	85	12–13
	C3H	20	15	75	14–57
	C3H presensitized to 101	20	0	0	18–51
	101	20	7	35	33–87
	101 presensitized to C3H	20	0	0	15–30
(101 × C3H)F_1	Radiation control	20	18	90	68
	C3H	20	10	50	52–90
	C3H presensitized to 101	20	0	0	28–77
	101	20	11	55	25–40
	101 presensitized to C3H	20	0	0	15–33

cells were suspended in Tyrode's solution according to the method described by Congdon and Urso(9). The tissues were chilled throughout the procedure. Some groups of (C3H × 101)F_1 and of (101 × C3H)F_1 mice received preimmunized parental liver cells; donor mice were preimmunized to the reciprocal parent of the F_1 combination by intraperitoneal implantation of 10 × 10^6 spleen cells 4 weeks before killing. Donor mice were killed by bleeding at the age of 3 to 5 months. In certain instances referred to, liver donors were anesthetized with Nembutal before killing and perfused with 20 ml of Tyrode's solution injected *via* the left cardiac ventricle. When liver donors were irradiated, the exposure conditions were identical to those described for the recipients. The term "frozen" liver indicates that the liver cell suspension was slowly frozen and kept overnight at –10°C before thawing and injection. No attempt was made to avoid the Eichwald-Silmser effect(10,11) by appropriate matching of donors and recipients according to sex and strain, but no evidence for sex-determined histoincompatibility was noted in the experiments with liver transplantation. For this reason, the sex of the donors and recipients is not indicated in the results presented.

Results. Effects on survival. All the 600-r irradiated (C57BL × 101)F_1 mice receiving parental strain liver cells died 12 to 25 days after treatment (Table I). In the second week after irradiation, the animals developed a condition resembling the foreign spleen reaction(5) showing weight loss, ruffling of hair, and atrophy of skin and lymphatic tissue. Both parental strains were effective in inducing the disease in 100% of the animals, although survival time after implantation of 101 liver cells was somewhat shorter. Of the irradiated F_1 control mice, all survived the observation period of 90 days with or without receiving isologous liver cells. Parental liver cells did not affect survival of nonirradiated F_1 hybrids. Mortality after inoculation of C3H liver cells into 600-r irradiated (101 × C3H)F_1 and (C3H × 101)F_1 mice was contrastingly low. The same F_1 hybrids showed a higher mortality, however, 80 to 90 days after inoculation of 101 liver cells. Liver cells from presensitized parental mice caused greater and earlier mortality among both experimental groups. Injection of lethally irradiated (900 r) mice with IBM did not alter the killing effect of parental liver in either strain combination (C57BL × 101)F_1 or (101 × C3H)F_1 (Fig. 1). At this higher radiation dose level, 0.1 equivalent of a mouse liver was enough to cause the disease in (C57BL × 101)F_1 mice. In the other hy-

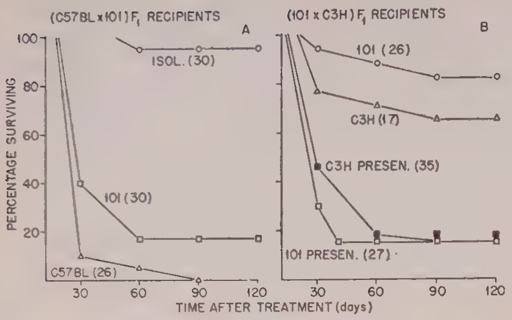

FIG. 1. Survival of 900-r irradiated F_1 hybrid mice inj. with isologous bone marrow (10×10^6 nucleated cells) and parental-strain liver cells (0.1 liver equivalent). Numbers in parentheses indicate No. of mice.

brid. unsensitized 101 and C3H liver cells gave rise to a cumulative mortality of about 25%. After presensitization of the donors, mortality rate increased markedly in the F_1 recipients, as it occurs in the corresponding sublethally irradiated groups. Isologous liver cells did not modify the acute radiation death pattern in lethally irradiated (C57 BL × 101)F_1 recipients. Inoculation of IBM into 600-r irradiated (C57BL × 101)F_1 mice prevented the killing effect of subsequently inoculated C57BL or 101 liver cells, at least during the first 90 days, in 74% of the treated animals. Mortality of the (C57BL × 101)F_1 recipients implanted with perfused C57BL liver cells occurred later than in those with liver cells from unperfused livers, but 100% mortality was nevertheless reached (Table II). Among the recipients implanted with 101 perfused liver cells, 15% are surviving after 90 days. When the parental liver was irradiated *in vivo* with 950 r of X rays within one hour before killing of the donor, or the cell suspension was frozen and thawed before injection, no mortality was observed in the irradiated F_1 hybrid recipients (Table II).

Pathological effects. Histological changes in the 600-r irradiated (C57BL × 101)F_1 hybrids injected with C57BL or 101 liver cells were found mainly in the hemopoietic and lymphoid tisues. Although hemopoietic elements in the white and red pulp of the spleen, in lymph nodes, and in bone marrow were actively recovering from radiation-induced atrophy by the second week, lymphoid follicles remained moderately atrophic. Six to 10 days after treatment, the lymphoid cells in the germinal centers were replaced with proliferating reticuloendothelial cells. This reaction was milder, however, than that after injection of parental spleen cells(5), even when the stimulus was exaggerated by injecting the equivalent of 2 livers per recipient. At time of death, the bone marrow was cellular; therefore, the immediate cause of death was not lack of bone marrow regeneration. In the 900-r irradiated F_1 mice infused with IBM and with parental liver cells, the red pulp of the spleen and the bone marrow were cellular at time of death. The lymphoid system was atrophic in all animals and follicles of the spleen and lymph nodes showed marked granulomatous proliferation of reticuloendothelial elements, similar to that described.

Discussion. Implantation of adult par-

TABLE II. Survival of 600-r Irradiated (C57BL × 101)F_1 Mice Injected with Parental Strain Liver Cells.

Donors	Treatment of donor	Treatment of liver	No. mice inj.	No. survivors	
				30 days	90 days
C57BL	None	None	40	0	0
101			40	0	0
(C57BL × 101)F_1			25	24	24
C57BL	”	Perfused with Tyrode's solution	20	7	0
101			20	7	3
C57BL	”	Frozen and kept overnight at −10°C	10	10	10
101			10	10	10
C57BL	950 r total-body 1 hr before killing	None	25	25	25
101			25	25	25
(C57BL × 101)F_1			10	10	9

ental-strain liver cells into (C57BL × 101)F_1 mice was followed by a fatal disease when the recipients were irradiated with a sublethal dose of X rays (600 r) or with a lethal dose (900 r) followed by subsequent inoculation of isologous bone marrow. Inasmuch as this effect was not caused by sublethal irradiation alone, by lethal irradiation and IBM infusion, or by irradiation and treatment with isologous F_1 liver cells, we infer that the observed reaction resulted from immunogenetic differences between F_1 hybrids and parental tissues. The similarity of these findings with those of the foreign spleen reaction(5,6) and the ability of transplanted liver cells to confer adoptive immunity(4) suggest that immunologically competent cells present in the grafted liver can give rise to an *in vivo* antigen-antibody reaction with consequent wasting disease.

The pathologic findings indicate that mice receiving parental liver cells do not die of bone marrow aplasia. Changes observed in spleen and lymph nodes, characterized by replacement of lymphoid elements with reticuloendothelial cells in the germinal centers, are similar to those observed during the foreign spleen reaction(5,6,12) and are of the same type, even if less pronounced, as the changes described by Congdon *et al.*(13) in lethally irradiated mice given homologous spleen cells intravenously.

Administration of adult parental liver cells to (C3H × 101)F_1, or reciprocal F_1, mice leads to different results probably because there is less pronounced immunogenetic difference between the latter two parents at the *H-2* and/or other loci(14), both parental strains being *H-2K* (R. D. Owen, personal communication). In this situation, parental liver cells failed consistently to provoke early and high mortality in irradiated F_1 recipients unless presensitized to the reciprocal parent of the hybrid. The mortality occurring after implantation of unsensitized 101 and C3H liver cells in 600-r irradiated F_1 mice is probably due to minor antigenic differences, reported also by Cosgrove *et al.*(15) in bone marrow transplantation experiments with the same mouse strains. A smaller amount of parental liver cells (0.1 liver equivalent) caused a mortality of about 25% in 900-r irradiated (101 × C3H)F_1 mice protected against radiation death with IBM. Also in the 900 r irradiated (C57BL × 101)F_1 hybrid 0.1 liver equivalent was enough to induce the wasting disease, whereas in 600-r irradiated F_1 recipients 0.25 equivalent was required. Radiation dose and liver cell dose are probably interrelated factors in determining the foreign liver reaction. The enhancement of the foreign liver reaction by presensitization of donors to the reciprocal parent denotes further the immunological nature of the reaction and suggests, according to Makinodan *et al.*(16) and to Cosgrove *et al.*(15), that the differences in immunologic effectiveness between various tissues reflect differences in number of existing antibody-forming cells.

By use of skin-grafting technics, commercially obtained strain 101 mice used in this laboratory have been shown to be incompletely homogeneous (Congdon, personal communication). But in these experiments, in which recipients were sublethally irradiated, if the 101 liver antigens were not identical with the 101 antigens of the (C3H × 101)F_1 or (C57BL × 101)F_1 constitution, the F_1 presumably should have rejected the liver, and deaths would not be expected. Data obtained with liver cells from perfused animals, after removal of circulating peripheral blood cells, showed that immunologically competent cells were still present in the liver in sufficient numbers to cause the graft-versus-host reaction. But their number was reduced, as one can argue from the delay in appearance of the killing effect and from the reduction in mortality It is possible that perfusion procedure not only removes the residual blood cells from the liver vessels but also some of the littoral reticuloendothelial cells. This does not exclude, of course, the possibility that the immunologically active cells might have been diffusely infiltrating lymphoid cells.

After having been frozen and thawed or after having been heavily irradiated *in vivo*, parental liver cells lost their ability to react immunologically in irradiated F_1 recipients. This is presumptive evidence that a viable cell was involved in the described foreign liver disease.

Protection of lethally irradiated (C57BL × 101)F$_1$ mice with IBM did not prevent the occurrence of the wasting disease after parental liver cell implantation. This finding could be easily explained by the assumed incapacity of the F$_1$ antibody-forming cells to react immunologically against parental antigens. In addition to this, it is well known that the antibody-forming cell content of normal mouse bone marrow tissue is very low (16). On the other hand, in sublethally irradiated F$_1$ hybrids, IBM infusion prevented the effect of parental liver cells and allowed 74% survival of the treated recipients; 100% survival was also noted when parental liver cells were introduced in nonirradiated F$_1$ hosts.

The need of irradiation for occurrence of a foreign spleen or liver reaction in a parent-to-hybrid combination has been related to lack of proliferation stimulus(5) and lack of proper space(6) for the grafted immunologically active cells in a nonirradiated adult recipient. Furthermore, it has been postulated that antibody-forming cells of certain genetic constitutions might be more or less sensitive to an excess of antigen(12) and sometimes be destroyed. The latter situation should not, however, be modified by recipients' whole-body irradiation. On the other hand, there is some evidence that the F$_1$ hybrid may react immunologically against parental-strain tissues. For example, Popp and Sapp(17) have observed spontaneous reversions in lethally irradiated (C57BL × 101)F$_1$ mice carrying an active transplant of C57BL bone marrow, and Cudkowicz *et al.*(18) have observed a high incidence of death (15 to 60 days after treatment) in (C57BL × 101)F$_1$ hybrids given a lethal whole-body radiation dose, parental bone marrow (C57BL or 101), and isologous liver cells. Also, the reduced incidence of foreign liver disease in 600-r irradiated (C57BL × 101)F$_1$ mice receiving isologous bone marrow could be explained in terms of a positive reaction of the F$_1$ against the parental tissues. That this reaction cannot be attributed to sex-determined histoincompatibility(10,11) between hybrid antibody-forming cells and parental liver antigen is evident from its occurrence even when F$_1$ recipient, F$_1$ bone marrow donor, and parental liver donor were females. The F$_1$-against-parent reaction could therefore occur in nonirradiated or in sublethally irradiated-isologous bone marrow restored animals of this strain combination but not after heavy irradiation of the F$_1$ recipient's immune mechanism.

Summary. 1. Implantation into irradiated (C57BL × 101)F$_1$ hybrids of viable liver cells from adult donor mice of the parental strains caused a wasting disease followed by death in over 90% of the recipients. 2. This "foreign liver disease" occurred after a sublethal dose of radiation was given to the recipients or after a lethal dose followed by infusion of isologous bone marrow. It did not occur in nonirradiated recipients but occurred in only 26% of sublethally irradiated F$_1$ hybrids infused with isologous bone marrow. 3. In a strain combination in which the F$_1$ hybrid was homozygous at the *H-2* locus, and the parents should therefore have been antigenetically relatively compatible, the "foreign liver disease" occurred late after treatment and was enhanced by presensitization of the donor to the reciprocal parent of the hybrid. It was shown that radiation dose and liver cell dose were inter-related factors in determining the development of the disease. 4. Deaths are ascribed to an *in vivo* immunologic reaction after antibody production by the grafted parental cells against F$_1$ antigens. Nevertheless, there is evidence of a possible immune response by the F$_1$ hybrid against the parent.

We are grateful to Mr. W. D. Gude and Mr. T. Mack for the histological preparations.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Growth of Laboratory and Naturally Occurring Strains of Eaton Agent in Monkey Kidney Tissue Culture. (26114)

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There has been renewed interest in the agent recovered in the embryonated egg from patients with atypical pneumonia by Eaton *et al.*,(1). Until recently considerable controversy has surrounded the role of this agent in respiratory illness. Studies carried out in the past few years, however, have provided evidence that Eaton agent is associated with human respiratory disease. Thus, 80 to 95% of patients with cold agglutinin positive atypical pneumonia were found to develop fluorescent stainable antibody for the agent (2,3). In addition, a controlled epidemiologic study in military recruits indicated that Eaton infection was associated with febrile upper respiratory illness as well as atypical pneumonia(4). Approximately 10% of children hospitalized with lower respiratory tract illness in Washington, D.C. developed a serologic response to this agent(5).

Early studies were hindered by the difficulty of working with an agent whose only index of activity was the production of lung lesions in a portion of, but not all, inoculated hamsters or cotton rats(1,6,7). Application of the fluorescent antibody technic resulted in a more quantitative assay system and permitted demonstration of specific antigen in the bronchial epithelium of the experimentally infected chick embryo(8). Employing this technic it was possible to recover a limited number of strains from patients with pneumonia by serial amniotic egg passage(3, 8,9). This procedure, however, is difficult, involved, and clearly limits the quantity of epidemiologic information which can be obtained. Simpler laboratory technics are needed to facilitate the recovery and identification of the agent before extensive field studies necessary to an understanding of the natural history of infection are possible.

This report will describe preliminary studies which indicate that Eaton agent propagates in several different types of tissue culture. In addition, it will be shown that naturally occurring strains of the agent can be recovered in monkey kidney tissue culture.

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‡ The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

Materials and methods. Eaton agent. The egg adapted laboratory strain designated FH was kindly supplied by Dr. C. Liu(8). This strain was passaged 4 times in yolk sac entodermal cultures at 7 to 9-day intervals in this laboratory, as described in a companion communication(10). A pool of the 5th, 7th, and 9th day harvests of the 4th passage constituted the inoculum used to initiate passage series in other culture systems.

Fluorescent antibody technic. This technic has been described previously(5). Chick embryo lung sections or tissue culture cover slips were routinely fixed in acetone at room temperature for 10 minutes. Human serum containing antibody to the Eaton agent was then placed on the slide and allowed to incubate at 37°C for 30 minutes. The slides were thoroughly washed in 3 changes of buffered saline (pH 7.2) following which goat anti-human globulin conjugated with fluorescein was then added. After 30 minutes at 37°C and 3 washes in buffered saline the tissue sections or cover slips were covered with buffered glycerol (pH 7.0) and viewed by UV microscopy.

Tissue culture. Rhesus monkey kidney and HEP-2 roller tube cultures, obtained from Microbiological Associates, were washed 5 times with Hanks' solution to remove antibiotics present in the growth medium. The cultures were maintained with Eagle's medium (containing double the usual quantity of amino acids and vitamins) and 5% inactivated chicken serum. Penicillin at a concentration of 2000 units per ml was the only antibiotic added to this fluid medium. Chick kidney cultures were prepared as described by Maassab and maintained with Eagle's medium and 5% chicken serum(11).

Titration. Tissue culture fluid or cell suspension was diluted in 4-fold steps in Eagle's medium with 5% chicken serum. One-tenth ml was then inoculated amniotically into each of ten 13-day-old embryonated eggs. After 6 days incubation at 35°C the surviving embryos were harvested and the thorax quick frozen in a CO_2 alcohol bath. Lung sections were prepared in the frozen state and then tested with a 1:20 dilution of a potent atypical pneumonia convalescent serum (titer of staining antibody = 1:320) for presence of specific antigen in the bronchial epithelium as described above. A minimum of 5 eggs were examined before a dilution of material was considered noninfective.

Collection of specimens. Throat swabs from patients with pneumonia were immersed in trypticase soy broth with 0.5% bovine albumin without antibiotics. The fluid was then quick frozen in a glass-sealed ampule and stored at –60° for 3 to 5 months.

Isolation attempts in eggs. Each of ten 13-day-old embryonated eggs was inoculated amniotically with 0.2 ml of throat swab fluid. After 6 days incubation at 35°C the lungs and trachea were removed from surviving embryos and ground in trypticase soy broth with 0.5% bovine albumin—2 ml per embryo. This suspension was then inoculated amniotically into ten 13-day eggs. After 3 such passages the lungs were quick frozen and examined for presence of specific antigen by the fluorescent antibody technic.

Isolation attempts in tissue culture. Six monkey kidney cultures were inoculated with 0.2 ml of throat swab fluid at the same time eggs were injected. Cultures were maintained at 35°C. Culture fluid was removed on the 7th and 11th days and stored at –60°C; fresh culture fluid was then added to the tubes. On the 15th day the monkey kidney cells and fluid were combined with the stored fluids and 0.2 ml of the mixture was inoculated into each of 10 eggs. After 6 days incubation, the lungs of the surviving eggs were harvested and tested for presence of specific antigen as described previously.

Results. Chick kidney cultures. Chick kidney cultures were 7 days old when inoculated with the 4th yolk sac culture passage of the FH strain. Fluid harvested on the 31st day of the 1st chick kidney passage contained Eaton agent, as shown by sub-inoculation into 13-day eggs. This represented a $10^{-7.0}$ dilution of the yolk sac culture inoculum when fluid changes over the 31-day period were considered. Twelfth day fluid from the 1st passage was used to initiate a 2nd

TABLE I. Multiplication of Eaton Agent in Monkey Kidney Tissue Culture.

	Reciprocal of egg infectious doses/0.1 ml of fluid or cell suspension at indicated time		
	Exp. 1	Exp. 2	
	Inoculum = 16 egg doses	Inoculum = 1000 egg doses	
Time after inoculation	Culture fluid	Culture fluid	Culture cells*
8 hr†	0‡	0	0
1 day	0	0	0
3 days	0	4	4
5 "	4	16	
7 "	4	256	
10 "	256 or >	1024	
15 "	256 or >	16384	16384

* Monkey kidney cultures were washed 3 times, the cells suspended in the same quantity of culture fluid used for maintenance—1.5 ml—and cell suspension frozen and thawed twice.

† Cultures were washed 3 times with 1.5 ml of Hanks' solution 6 hr after inoculation following which 1.5 ml of maintenance medium was added to each tube.

‡ A minimum of 5 inoculated eggs was tested before a specimen was considered negative.

passage in chick kidney culture. Fluid harvested on the 7th day of the 2nd passage contained $10^{1.8}$ egg infectious doses of the agent. This represented a $10^{-7.6}$ dilution of the yolk sac culture inoculum. These findings suggest that the agent replicated in this culture system.

Specific antigen, however, was not visualized when infected cover slip preparations were tested by the fluorescent antibody technic. These tests were performed with a 1:10 dilution of an atypical pnumonia convalescent serum which had a staining titer of 1:320 to 1:640 with infected chick embryo lung sections. No cytopathic effects were observed.

HEP-2 culture. The 4th yolk sac culture fluid was inoculated into HEP-2 cultures and the agent was recovered after 31 days at 35°C, representing a $10^{-8.4}$ dilution of the inoculum. A 2nd successful passage was carried out in HEP-2 cells. No cytopathic effects were observed.

Monkey kidney. A passage series in rhesus monkey kidney culture was initiated with 4th yolk sac passage material. At the end of 3 serial transfers at 7 to 10 day intervals, culture fluid contained $10^{4.2}$ egg infectious doses, representing a $10^{-14.2}$ dilution of the yolk sac culture inoculum. This indicated that Eaton agent replicated in monkey kidney tissue culture.

Growth of the agent in monkey kidney was then studied in a more detailed manner since the level of infectivity attained in this system was higher than that observed in yolk sac, chick kidney, or HEP-2 cultures. The results of 2 growth curve experiments are shown in Table I. In these experiments the cultures were washed 6 hours after inoculation to reduce the quantity of unadsorbed infectious material. Employing this procedure it was possible to demonstrate an eclipse phase which lasted 1 to 3 days, depending on size of the inoculum. A high level of infectivity did not develop until the 10th to 15th day.

Tissue culture cells were thoroughly washed, then frozen and thawed twice in Exp. 2 to assay infectious material associated with the cell. The data suggest that Eaton agent was not retained in association with the cell for a long period since early in infection and later at time of peak production the quantity of infectious material associated with cells was the same as that found in the fluid medium.

Although Eaton agent multiplied to a level of $10^{4.2}$ egg infectious doses in monkey kidney cells, specific antigen was not visualized by means of indirect immunofluorescence employing a potent human serum at a 1:10 dilution. Infected tissue culture cover slips also were fixed by other methods (acetone at −60°C for 2 hours, ethanol at room temperature, and ethanol at −60°C for 2 hours) which permitted staining of chick embryo lung antigen but specific fluorescence was not observed. Cytopathic effects were not seen in infected cultures. Elementary bodies or inclusions were not seen when infected cover slips were stained with Macchiavello or Giemsa stain. (The tests were performed through the courtesy of Miss E. B. Jackson).

Recovery of the agent in monkey kidney culture. An opportunity to test the efficacy of monkey kidney cultures for recovery of naturally occurring strains of Eaton agent was afforded during an epidemiologic study of infection among marine recruits(4). Throat swab fluids from individuals with pneumonia who developed fluorescent stainable antibody during convalescence were inoculated into monkey kidney culture. Recovery of an agent in culture was determined by sub-inoculation of the combined 7, 10, and 15-day fluids into eggs which were tested for antigen by the fluorescence technic. Fourteen strains were recovered in culture from 17 patients with pneumonia. One tissue culture isolate was passaged twice more in monkey kidney cells and on the 13th day of the 3rd passage the fluid medium contained $10^{3.0}$ egg infectious doses, representing a $10^{-10.4}$ dilution of the original throat swab fluid. This suggests that the isolate propagated in monkey kidney cells and that the positive result in eggs did not represent mere persistence of the agent in the culture fluid.

Although throat swab fluids were inoculated simultaneously into tissue culture and eggs only 6 specimens were suitable for comparative study because of the problem of bacterial contamination during amniotic passage. All of the 6 specimens were positive in tissue culture whereas only 5 yielded an isolate when tested in eggs, suggesting that the culture system was as sensitive as the hen's egg to naturally occurring strains of the agent.

Strains. Three of the tissue culture isolates were compared among themselves as well as with the FH strain to determine if antigenic differences existed. Sections of lung from chick embryos infected with the various strains were employed in tests with the acute and convalescent sera of the individuals from whom the isolates originated. The 3 tissue culture isolates were similar and did not differ from the FH strain recovered by Liu (Table II)(8).

TABLE II. Antigenic Relationship of Strains Recovered in Tissue Culture to F.H. Strain of Eaton Agent.

	Reciprocal of fluorescent antibody titer with indicated strain			
		Strains recovered in tissue culture		
Convalescent phase serum*	F.H.	P.I. 646	P.I. 725	P.I. 898
Parris Island #646	40	40	40	40
” ” 725	80	40	40	160
” ” 898	160	160	80	160

* Acute phase serum gave negative (<1:10) results when tested with all 4 strains.

Discussion. The results of this study suggest that the Eaton agent multiplies in chick kidney, HEP-2, and monkey kidney tissue cultures. Growth of the agent has also been demonstrated in yolk sac entodermal cultures (10). Of some interest was the demonstration of an eclipse phase during replication in monkey kidney cells. This property suggests that the agent should be classified as a virus according to a recent definition proposed by Burnet(12). Large size (180 to 250 mμ) of the Eaton agent and its sensitivity to the antibiotics streptomycin and aureomycin, however, poses some difficulty in regard to such a classification(6,13,14,15). A similar difficulty exists among the psittacosis-lymphogranuloma agents, a group which is biologically but not antigenically similar to the Eaton agent.

The purpose of this investigation was to develop simpler technics for recovery and identification of the agent, to facilitate epidemiologic studies. This aim was only partially realized. It was shown that monkey kidney tissue culture was as sensitive to naturally occurring strains of Eaton agent as was the hen's egg. One egg passage and immunofluorescent examination of chick embryo lung sections were still necessary, however, to determine presence and quantity of infectious material in the culture system. Attempts to visualize specific antigen in infected monkey kidney cells were unsuccessful, presumably because of an insufficient concentration of antigen. Efforts in this direction as well as attempts to obtain a culture system in which cytopathic effects occur are clearly indicated and are continuing.

The 3 isolates from 1959 selected for characterization were found to be antigenically similar and to resemble the FH strain recovered in 1954(8). Previous studies indicated that the FH strain was indistinguishable from strains recovered in 1943, 1944, 1954, 1955, 1956, and 1958(2,3). These findings suggest that the Eaton agent has a stable antigenic configuration.

Summary. An egg adapted strain of Eaton agent multiplied in rhesus monkey kidney tissue culture. Antigen concentration within the cells was insufficient to permit visualization by immunofluorescence. An eclipse phase was demonstrated during replication in monkey kidney cells. In a simultaneous test monkey kidney culture was found to be as sensitive as embryonated eggs for recovery of naturally occurring strains of Eaton agent. Fourteen strains were recovered in tissue culture from 17 patients with pneumonia.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Growth of the Eaton Agent of Primary Atypical Pneumonia in Chick Entodermal Tissue Culture.* (26115)

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The Eaton agent, associated with primary atypical pneumonia, has had a limited range of known susceptible host cells(1,2). Although pneumonia may appear after intranasal inoculation of hamsters or cotton rats, its irregularity renders precise experiments difficult. Liu(3) developed a dependable, although cumbersome, laboratory method of demonstrating the agent by using its infectivity for bronchial epithelium of the embryo chick in conjunction with indirect fluorescent antibody staining. It is clear that acquisition of additional information about this agent depends to a great extent upon improved methods of study.

The investigation described here was undertaken with the thought that cultures of entodermal cells taken early from chick embryos might prove to be susceptible to this agent since it can infect an entodermal derivative in the older embryo. The methods used followed closely those described for cultivation of rickettsiae and psittacosis group viruses in chick entodermal cell cultures(4,5). The Eaton agent was shown to multiply in such cultures by successfully accomplishing 10 serial passages in one series, and 6 in another.

Materials and methods. The FH strain of Eaton agent, kindly supplied by Dr. Chien

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* The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

Liu, was used. For the first tissue culture inoculation a preparation of infected chick embryo lung and trachea was subjected to a partial purification procedure previously used for chick embryo yolk sacs infected with rickettsiae or psittacosis viruses(4). The method includes digestion of the crude emulsion with trypsin, sedimentation of the infective particles by centrifugation, resuspension in Hanks' balanced salt solution (HBSS), and further clarification by addition of bovine plasma albumin to 0.6% concentration, and celite to 10%, followed by light centrifugation. The supernatant, containing the infectious particles, is relatively free of tissue components.

The entodermal cell cultures were prepared from explants from the blastoderm of 4-day chick embryos, and grown on circular coverslips in flat-bottomed tubes(4). The medium consisted of HBSS, 75%, and chicken serum, 25%; it was used in a volume of 0.5 ml per tube. Inoculation was accomplished by replacing with a similar medium to which the infective preparation was added in 10% dilution. Incubation was at 36-37°C. Two hundred units of penicillin per ml were incorporated with the medium in the first 5 passages of Series A; the last 5 passages contained no antibiotic. Penicillin at 2000 u per ml was employed throughout for the 6 passages of Series B. Ten cultures were routinely used for each passage and uninoculated controls were regularly included.

The fluorescent antibody technic has been described previously(6). Chick embryo lung sections or tissue culture coverslips were routinely fixed in acetone at room temperature for 10 minutes. A 1:10 dilution of a human serum containing antibody to the Eaton agent was then placed on the slide and allowed to incubate at 37°C for 30 minutes. The convalescent sera used had a staining antibody titer of 1:320 to 1:640. Acute phase (negative) sera from the same patient were also used for control purposes.

Results. Two series of passages were accomplished by transfer of fluid from infected cell cultures. One series was carried out in each of the two laboratories represented, but both were initiated with the same preparation of partially purified infected chick lung.

Series A (10 passages). The indirect method of staining with fluorescent antibody was applied to both infected and control coverslip cultures from the first 4 passages, taken at various intervals after inoculation, and at the 11th day in the 10th passage. Specific fluorescence was not seen with certainty, presumably due to insufficient concentration of specific antigen in the cells. However, the agent was demonstrated in culture fluid repeatedly throughout the series by infection of chick embryos and detection of the agent in the chick lung by fluorescent antibody staining. Examination of cultures after staining by May-Greenwald-Giemsa revealed no cytopathic effects.

Centrifugation of infective particles onto chick entodermal cells has been found to increase greatly the infectivity of rickettsiae and psittacosis viruses for chick entodermal cells(4). The reported large size of the Eaton agent, 180-250 mμ (2, see also 7), suggested that this maneuver might be advantageous. The first passages included groups of inoculated tubes which were centrifuged at 20°C for one hour at 1800 g. It was soon found, however, that passage was successful without centrifugation and the practice was discontinued.

The second and third passages were made with fluids harvested on the 6th day, and thereafter with fluids taken on the 10th or 11th day. This was usually preceded by a change of medium on the 5th day. In the final (10th) passage both 5-day and 11-day fluids were tested but only the latter was found infective, suggesting a higher level of infectivity at the latter interval. The 10-fold dilution effected at each passage gives an estimated 10^{-10} dilution of the original inoculum in the final 10th passage fluid. This figure can be further reduced by approximately 5 log units because of the complete fluid change made once in each of 5 passages. Detection of infectivity in the 10th passage indicates clearly that increase of the agent occurred in this culture system.

Series B (6 passages). These passages

were made at 7 to 9-day intervals, and fluid on the infected cultures was frequently changed once or twice in the interim. On the 14th day of the 6th passage the original inoculum had been diluted 10^{-11} by the transfers and fluid changes. Fluid harvested at that time was infective for 13-day embryonated eggs as demonstrated by fluorescent antibody staining of lung sections, indicating that the agent had replicated in the entodermal cell cultures. The agent was still present in fluids removed from cultures at 16-18 days of incubation. Specific antigen, however, was not visualized when infected coverslip preparations were tested by the fluorescent antibody technic and no cytopathic effects were observed.

Comments. Chick embryo lungs infected with the FH strain may contain 10^5 or 10^6 embryo infective doses per 0.2 ml(3). Although the original inoculum in these series was not titrated, the finding of the infective agent after 10 and 6 passages, in the 2 series, at estimated dilutions of the original inoculum of 10^{-11} or greater, leaves no doubt that the agent increased in the chick entodermal cell cultures. No titrations of the culture harvests were made and there is no evidence that any degree of adaptation to these cells occurred. The identity of the agent cultured was established by characteristic appearance after fluorescent staining of subcultures in embryo lung with known antisera (convalescent), and the more extensive serologic examination applied to later cell cultures infected by passage from entodermal cells, as described in the accompanying paper(8). Unfortunately, this method of cultivation in entodermal cells does not provide any improvement in current technics. However, this first step in propagation of the Eaton agent in cultured cells suggests that better *in vitro* methods can be found.

Summary. The Eaton agent of primary atypical pneumonia was carried through 2 series of 10 and 6 passages respectively, in chick entodermal cell cultures, indicating growth of the agent in this cell system. Cytopathic effects were not seen and fluorescent antibody staining of the infected cells by the indirect method was negative. The agent was demonstrated in fluids from the infected cultures by subinoculation to chick embryos and fluorescent antibody staining of lung sections.

The authors gratefully acknowledge the technical assistance of George W. Gary, Jr., and Luther B. Townley.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Oxidative Phosphorylation and Amino Acid Incorporation into Protein in Regenerating Rat Liver. (26116)

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It has been shown that incorporation rate of labeled amino acids into protein(1-4) and oxygen consumption(5,6) is higher in the regenerating than in the normal rat liver. Since in this condition, incorporation of amino acids into protein depends upon aerobically formed high energy phosphate(7), it is possible that the increased oxidative proc-

TABLE I. Oxygen Consumption, P/O Ratio and Incorporation of C^{14}-Labeled Amino Acids into Protein of Normal and Regenerating Rat Liver. No. of experiments in parentheses. Mean values ± S.E.

Hr after hepatectomy	Q_{O_2} (slices)	P/O (homogenate)		Incorporation into protein (slices) (μmoles/g protein/hr)		
		Succinate	α-Ketoglutarate	Glycine-1-C^{14}	L-leucine-C^{14}	L-phenylalanine-C^{14}
0‡	5.06 ± .22 (24)	1.87 ± .05 (6)	3.56 ± .12 (19)	3.19 ± .26 (8)	2.63 ± .05 (8)	1.30 ± .11 (8)
84	5.82 ± .28* (24)	1.28 ± .06† (5)	2.39 ± .11† (37)	5.56 ± .40† (8)	4.34 ± .21† (8)	3.38 ± .31† (8)
168	6.20 ± .24† (24)	1.57 ± .19 (6)	3.21 ± .12* (17)	5.84 ± .46† (8)	3.36 ± .15† (8)	2.39 ± .21† (8)
240	5.45 ± .24 (24)	1.91 ± .04 (7)	3.71 ± .09 (18)	4.28 ± .22† (8)	3.23 ± .15† (8)	1.54 ± .07 (8)

* $p < .05$, compared to normal liver. † $p < .01$, compared to normal liver. ‡ Control.

esses may result in a proportional increase in ~ P which, in turn, could support a faster rate of protein synthesis by the multiplying hepatocytes. To investigate this possibility, oxygen consumption, oxidative phosphorylation and incorporation of some amino acids into protein of regenerating rat liver were measured.

Methods. Wistar rats of either sex, weighing about 250 g were partially hepatectomized according to the technic of Higgins and Anderson(8). After a 12-hour fast, normal and hepatectomized rats were decapitated 84, 168 and 240 hours after operation and their livers were removed. Oxidative phosphorylation, amino acid incorporation into protein and oxygen consumption were measured on separate fractions of liver as follows: *1. Oxidative phosphorylation.* The phosphorylation quotient (P/O ratio), with succinate and α-ketoglutarate as substrates, was determined according to the procedure described by Krebs *et al.*(9), based on simultaneous measurement of incorporation rate of labeled inorganic phosphate into ATP and of oxygen consumption by tissue homogenates. Phosphate fractions were separated by paper chromatography(10) and ATP turnover rate was calculated with the formula proposed by Krebs *et al.*(11). Radioactivity was measured with a liquid type Geiger-Müller counter. *2. Incorporation of amino acids into protein and oxygen consumption.* About 50 mg of liver slices were incubated in Warburg flasks for one hour at 38°C in air and in a medium containing NaCl, 1.28×10^{-1} M; KCl, 1.35×10^{-2} M; $CaCl_2$, 1.94×10^{-3} M; $MgSO_4$, 6.8×10^{-4} M; Na phosphate buffer, 1×10^{-2} M (pH 7.2); and either glycine-1-C^{14}, L-leucine-U-C^{14} or L-phenylalanine-U-C^{14} 1×10^{-3} M (the amino acid was added after 10 minutes of equilibration). After incubation the flasks were quickly chilled in an ice bath, the slices were removed and the proteins were purified as described previously (4). Samples were plated and counted using a mica window Geiger-Müller counter. Oxygen consumption of liver slices was measured in an identical system, except without added amino acids, using the standard Warburg technic(12).

Results and discussion. Results are shown in Table I. A moderate and statistically significant increase in oxygen consumption by regenerating liver slices occurs 84, 168 hours after partial hepatectomy and can still be seen 240 hours thereafter, although at this time it is no longer significant. This increase is of the same order of magnitude of that previously reported(5,6). A statistically significant lowering of the P/O ratio occurs 84 hours after partial hepatectomy, using either succinate or α-ketoglutarate as a substrate. When α-ketoglutarate is used, the ratio is still below normal 168 hours after operation, although, at this time, using succinate, the decrease is no longer significant. Two hundred and forty hours after operation, P/O ratio returns to normal with either substrate. In partial agreement with our results, Palladina and Gudina(13) reported a P/O ratio slightly lower than normal, using succinate, guanine and xanthine as substrates, 7 days after hepatectomy. The coupling between oxidation and phosphorylation is a labile mechanism strictly dependent upon the integrity of the mitochondria(14). Since the regenerating hepatocytes easily undergo fatty infiltration and cell vacuolation, it is possible that these regressive processes may bring about sufficient mitochondrial damage to cause the observed decrease in P/O ratio. This hypothesis is consistent with the observation that during liver regeneration, the activity of certain mitochondrial enzymes such as glutamic dehydrogenase(15), succinic dehydrogenase and cytochrome oxidase(5) decreases more markedly than would be expected from the decrease in mitochondrial population(16). The rates of glycine-1-C^{14}, L-leucine-C^{14} and L-phenylalanine-C^{14} incorporation into proteins are markedly enhanced in the regenerating liver in comparison to the controls. The difference is statistically significant 84, 168 and 240 hours after partial hepatectomy, except for the increased incorporation of phenylalanine which lasts only 168 hours. This marked increase in protein synthesis occurs simultaneously with a moderate increase in oxygen consumption and a decrease of P/O ratio, a situation in which the formation of high energy phosphates not only does not increase, but may actually decrease. These results suggest that, in the normal liver, the availability of ~ P greatly exceeds that needed for protein synthesis, as shown also by the calculations of Quastel and Bickis(7). In the normal liver, the excess of available energy could be utilized for other hepatic functions, whereas, in the regenerating liver, a greater part of energy would be used to support the increase in protein synthesis and other endergonic processes such as deoxyribonucleic, ribonucleic acid(17) and phospholipid(18,19) synthesis.

It should be noted that cellular multiplication is not the only condition in which an increased amount of energy is channeled toward synthetic processes, since an increased incorporation of amino acid into protein accompanied by normal oxygen consumption and moderate uncoupling of oxidative phosphorylation, has been described also in a regressive process, such as cloudy swelling(14,20). In addition, if we assume that the relative amounts of labeled glycine, leucine and phenylalanine incorporated into liver proteins correspond to the proportions of the 3 amino acids in these proteins, our results suggest that no striking changes in liver protein composition take place during the process of parenchymal regeneration, at least as far as these 3 amino acids are concerned. This is in agreement with previous results demonstrating the similarity between the electrophoretic pattern of soluble proteins(21,22) and the immunologic specificity of mitochondrial and microsomal proteins(23) obtained from regenerating and normal rat liver.

Summary. In slices from regenerating rat liver 84 and 168 hours after hepatectomy, oxygen consumption is moderately increased and glycine-1-C^{14}, L-leucine-C^{14} and L-phenylalanine-C^{14} incorporation into protein is markedly increased as compared with normal liver slices. On the other hand, in liver homogenates, incubated with succinate or α-ketoglutarate as substrates, oxidative phosphorylation is decreased in the early stages (84 hours) of regeneration. The results suggest the following conclusions: 1) in regenerating liver tissue, amino acid incorporation into protein can increase even without a parallel increase in the aerobic formation of high energy phosphates; 2) in both normal and regenerating rat liver, the availability of ~ P greatly exceeds that needed for protein synthesis.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Effect of Dietary Proline and Hydroxyproline on Tensile Strength of Healing Wounds.* (26117)

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The deleterious effects of ascorbic acid deficiency and protein depletion on collagen synthesis in healing wounds have been well established. Analysis of tissue from the wounds of scorbutic animals reveals abnormally high levels of the amino acid-proline and abnormally low levels of hydroxyproline (2). The reversal of the normal hydroxyproline-proline ratio in scurvy and the finding of low hydroxyproline levels in protein depletion states have suggested that an inability to hydroxylate proline may be an important factor in the retarded synthesis of collagen in both conditions(11). Abnormalities in the ground substance, such as a reduction in the acid mucopolysaccharides, may also be responsible for the failure to develop normal tensile strength which requires, in addition to collagen synthesis, that newly created tropocollagen particles be organized into purposeful patterns. However, if there were not serious fundamental defects in the intracellular synthesis of the collagen molecule, one would expect to find normal proline-hydroxyproline ratios in the wound tissues because the amino acid composition of tropocollagen is essentially the same as that of polymerized or mature collagen(6).

We have postulated that, if there is a defect in synthesis of collagen by fibroblasts in scorbutic or protein depleted animals (presumably at the hydroxylation of proline stage), tensile strength of their wounds might be favorably influenced by supplementing their diets with pure synthetic hydroxyproline. Previous investigations of the metabolism of hydroxyproline by Stetten(3), Green and Lowther(9), and Wolf and Berger(10) strongly suggest that free hydroxyproline probably would not be utilized in production of collagen during healing. In normal animals there is very little incorporation of dietary hydroxyproline in the general protein pool; nearly all of N^{15} labeled hydroxyproline can be recovered in the urine and stools (Stetten(9)). However, there is an extremely low turnover of the amino acids in a normal animal's collagen (Neuberger and Slack(8)), therefore Stetten's experiments do not necessarily mean that an animal which was actively attempting to synthesize new collagen under the handicap of protein or scorbutic acid deficiency would not be able to by-pass the hydroxylation of proline and utilize free hydroxyproline for collagen synthesis. Green and Lowther(3) investigated the possibility of incorporating free N^{15} labeled hydroxyproline into collagen which was being formed by an *in vitro* tissue slice from a carrageenin granuloma. Their results showed that practically all of the hydroxyproline was produced by hydroxylation of bound proline and that almost none of the free labeled hydroxyproline was incorporated in the saline extractable or new collagen fraction. They showed, however, that fibroblasts' cell membranes were freely permeable to hydroxyproline, therefore we still wondered if free hydroxyproline could be utilized by fibroblasts in healing wounds.

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* Supported by grant from Nat. Inst. of Arthritis and Metab. Dis., U.S.P.H.S., Bethesda, Md.

Because Green and Lowther also showed that the hydroxyproline in newly formed collagen was derived from free labeled proline, we also thought it worthwhile to determine whether administration of proline to protein depleted rats would influence rate of collagen production.

Methods and materials. 1. Two hundred and twenty-four Sprague Dawley female rats weighing between 165 and 190 g were divided into 4 groups of 56 rats each. The first group was fed a standard rat chow. Group 2 was offered 15 g daily of a 5% casein protein depletion diet with full vitamin supplements. The rats in Group 3 received a 5% casein protein depletion diet to which .5% d-l hydroxyproline had been added. This amount of hydroxyproline was selected on the basis of experiments by Mitoma and Udenfriend which showed that approximately one-sixth of this amount of 4-keto-l-proline would double the blood level of free hydroxyproline for 4 hours(7). The rats in Group 4 received a 5% casein protein depletion diet to which 1% d-l synthetic proline had been added. This amount of proline approximately replaced the proline in the protein depleted diet to normal levels. All of the rats were given tap water *ad lib.*

Amount of food consumed each day was calculated by weighing the uneaten residuum. Rats in Groups 3 and 4 were pair fed with the rats in Group 2 on the following day. After a period of 6 days to allow the rats to become accustomed to their diets, they were anesthetized with ether and the hair over the back of the neck and shoulders was clipped. A 2 cm longitudinal incision was made through the skin and subcutaneous fat midway between the shoulders. The wounds were closed immediately with four #40 steel wire sutures.

Eight rats in each group were sacrificed at 2-day intervals between the 6th and 21st days after wounding. A 1 cm segment of the wound was excised and tensile strength of the scar tissue was determined by the method of Williamson(1). Results of the tensile strength determinations were recorded and expressed as number of grams of water which exerted a force on the wound sufficient to cause a sudden complete separation of the wound edges. Such an endpoint was quite definite in most of the wounds tested.

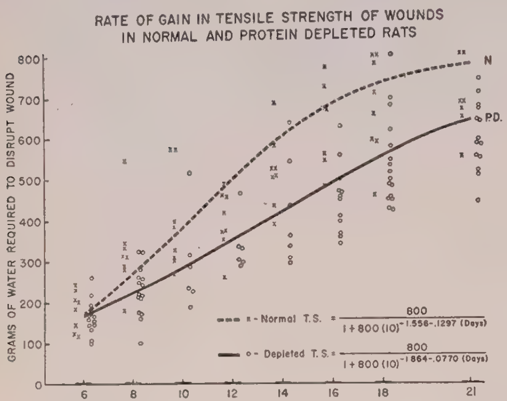
2. One hundred and fifty-eight female guinea pigs weighing between 250 and 300 g were divided into 3 groups of 56 each. Group 1 was fed a normal diet. Group 2 was fed an ascorbic acid deficient diet. Group 3 was fed an ascorbic acid deficient diet and also received a .5% hydroxyproline dietary supplement. The guinea pigs were wounded and tensile strength of the resulting scar tissue was determined as in 1.

Results. Fig. 1 shows a highly significant 1% level reduction in rate of gain in tensile strength in wounds of depleted animals. The data also reveal that rats fed a 5% casein protein-depletion diet did not show significant reduction in the strength of their wounds during the pre-collagen (0-5 day) period and that ultimately the wounds become as strong as those of normal animals.

Fig. 2 compares rates of healing of normal, protein depleted, protein depleted-hydroxyproline supplemented, and protein depleted-proline supplemented rats.

Apparently, the rate of gain of tensile strength in the wounds of rats receiving hydroxyproline supplement is not statistically significant. Neither proline nor hydroxyproline influenced rate of gain of tensile strength in protein depleted rats.

Fig. 3 compares rates of gain of tensile strength in the wounds of normal guinea pigs, scorbutic guinea pigs, and scorbutic pigs receiving a .5% hydroxyproline dietary supplement. It was difficult to keep scorbutic guinea pigs of this size alive longer than 3 weeks—therefore the number of determinations is smaller than in Fig. 1. Hydroxyproline supplement did not aid healing in the scorbutic guinea pigs.



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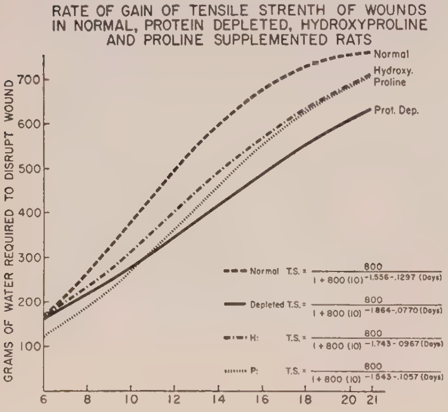

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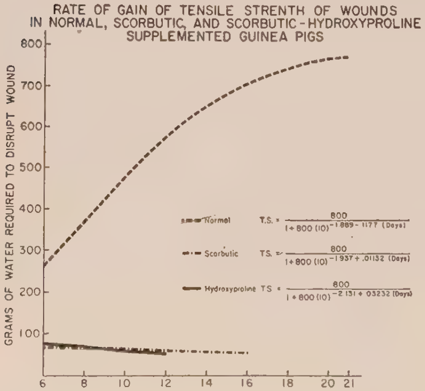

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FIG. 1-3.

Discussion. Stetten's dietary experiments with intact rats led her to conclude that, "The hydroxyproline of proteins is not derived to any appreciable extent from dietary hydroxyproline, but rather from the oxidation of proline which is already bound, presumably in peptide linkage(3)." Green and Lowther, working with *in vitro* tissue slices of carrageenin granuloma, reported that free hydroxyproline was not incorporated into new collagen fibers(9). Depletion and ascorbic acid deficiency reveal that the results of Stetten and Green also apply to the wounded animal, and that impaired wound healing in pathological states cannot be overcome by administration of dietary hydroxyproline. It is interesting to note, however, that abnormally high levels of serum hydroxyproline did not cause a reduction in tensile strength of healing wounds; apparently there was no interference with formation of intermolecular hydrogen bonds between the hydroxyl group of the hydroxyproline and the keto group of the peptide link within the collagen fiber. Competitive inhibition might have been anticipated at this point as suggested by Gross and Kirk(4).

Summary. Protein depleted rats were given dietary supplements of .5% synthetic d-l hydroxyproline and 1% synthetic d-l proline. Guinea pigs on an ascorbic acid deficient diet were given a dietary supplement of .5% d-l hydroxyproline. The animals were wounded by a standard technic and the tensile strength of their healing wounds was tested at 48-hour intervals between the 6th and 21st postoperative days. Neither hydroxyproline nor proline exerted a significant effect upon the rate of gain of tensile strength in the wounds of protein deficient rats or scorbutic guinea pigs.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Inhibitory Effect of Bicarbonate on ATP Levels and Cholesterol Biosynthesis in Liver Homogenates.* (26118)

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Rat liver homogenate preincubated with protamine loses the capacity to convert mevalonic acid (MVA) to non-saponifiable material (NSF, largely cholesterol)(1). This loss of synthetic capacity is associated with the absence of ATP in these homogenates. ATP is present and conversion of MVA to NSF occurs if the homogenates are supplemented prior to incubation with RNA, DNA or polyethylene sulfonate. Thus it would appear that there is a requirement for a relatively non-specific polyanion that is essential for maintenance of ATP levels in liver homogenates. During studies on the specificity of protamine as an inhibitor of respiration or of oxidative phosphorylation it was observed in a number of experiments that protamine that had been trypsinized in sodium bicarbonate solution was no longer deleterious but the sodium bicarbonate solution in which protamine had been trypsinized was quite inhibitory. More extended study has now shown that bicarbonate is a potent inhibitor of the aerobic system concerned with maintenance of ATP levels in homogenates and that trypsinized protamine, amino acids of the urea cycle, or canavanine, for reason to be discussed later, reverse, at least over a narrow range, this inhibition. In this connection the studies of Miller and Evans who showed that bicarbonate is an inhibitor of cytochrome oxidase are pertinent(2).

Methods and materials. The biosynthetic experiments involved preincubation of 5 ml aliquots of a 200 xg supernatant fraction of rat liver homogenate prepared as previously described(3,4,5) with 6 ml amounts of solution containing 1 mg ATP, 10 mg DPN, 100 mg sodium succinate, various amounts of sodium bicarbonate, and other supplements to be studied. Each flask was aerated with a stream of oxygen and preincubated with agitation for 30 min at 37°. Following preincubation each flask was opened and 1 ml MVA-2-C^{14} solution added, the contents aerated and the flasks then reincubated for an additional 3 hr. After final incubation, homogenates were saponified, extracted with petroleum ether, the ether extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture, and counted. In experiments involving a determination of ATP content of homogenates at time of MVA addition following preincubation the contents of paired flasks were treated with an equal volume of cold 4% perchloric acid. The precipitate that formed was removed by centrifugation and the supernatant solutions were freed of excess perchloric acid by careful neutralization with cold 10% potassium hydroxide.

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* Supported by research grants from Nat. Science Foundation and Nat. Inst. Health and by funds made available through State University of New York.

TABLE I. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Various Sodium and Potassium Salts.

Exp.	Level of material tested, mg/flask	NSF, cpm
1		7242
	10 $NaHCO_3$	7716
	20 "	8922
	30 "	6128
	50 "	21
	10 NaCl	7919
	20 "	7522
	30 "	6873
	50 "	6740
2		5825
	10 $NaHCO_3$	7123
	20 "	7731
	50 "	28
	10 $KHCO_3$	6359
	20 "	8240
	50 "	20
	10 NaCl	5955
	20 "	6094
	50 "	5631

The solutions were centrifuged to remove potassium perchlorate and the supernatant solutions were subjected to anion exchange chromatography and gradient elution on Dowex-1 as described by Allfrey and Mirsky (6). ATP was determined strectrophotometrically in the eluates. Protamine sulfate was obtained from the Mann Research Laboratories. The amino acids of the urea cycle and canavanine were all of the L form and were obtained from Nutritional Biochemical Corp. or California Corp. for Biochemical Research.

Results. The results summarized in Fig. 1 show that biosynthesis of NSF from MVA by liver homogenates is completely inhibited by preincubation with about 0.05 M sodium bicarbonate. Note in particular the steep slope of the curves at levels of sodium bicarbonate just below those showing complete inhibition. Levels of sodium bicarbonate of about 0.02 M are stimulatory. Separate studies have established that the inhibitory effect of sodium bicarbonate in the amounts studied is not attributable to alterations in the pH of the homogenates (no detectable change in pH with up to 50 mg of sodium bicarbonate per flask).

Fig. 2 shows that a control homogenate of liver preincubated without bicarbonate maintained an adequate level of ATP as determined by anion exchange chromatography on Dowex-1 formate. Biosynthesis of NSF by homogenate in a paired flask supplemented with MVA following the preincubation period was excellent (7505 cpm). On the other hand, homogenate of liver preincubated with 0.05 M bicarbonate did not maintian a level of ATP and biosynthesis of NSF in a paired flask supplemented with MVA following the preincubation period was negligible (20 cpm).

Biosynthesis of NSF from MVA is not affected by preincubation of homogenates with sodium chloride (Table I). Preincubation with potassium bicarbonate is accompanied by increased activity of the system at intermediate levels and by complete inhibition at high levels as shown previously with sodium bicarbonate.

The data of Table II demonstrate that protamine digested with trypsin is active in reversing the inhibitory effect of sodium bicarbonate. Protamine freshly dissolved in buffer or protamine digested in bicarbonate without trypsin is inactive. Separate studies showed that the effect of trypsinized protamine is not due to the trypsin. It should be pointed out that the results summarized in Table II were obtained only when the level of sodium bicarbonate used was just maximally inhibitory. At higher levels of sodium bicarbonate no reversal of inhibition was ob-

TABLE II. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Sodium Bicarbonate and Various Protamine Preparations.

$NaHCO_3$, mg/flask	Additional supplement, mg/flask	NSF, cpm
		6950
40		25
"	4 Protamine	21
"	8 "	15
"	12 "	27
"	20 "	19
"	4 Protamine, incubated	28
"	8 *Idem*	29
"	12 "	21
"	20 "	18
"	4 Protamine, incubated with trypsin	23
"	8 *Idem*	66
"	12 "	222
"	20 "	6312

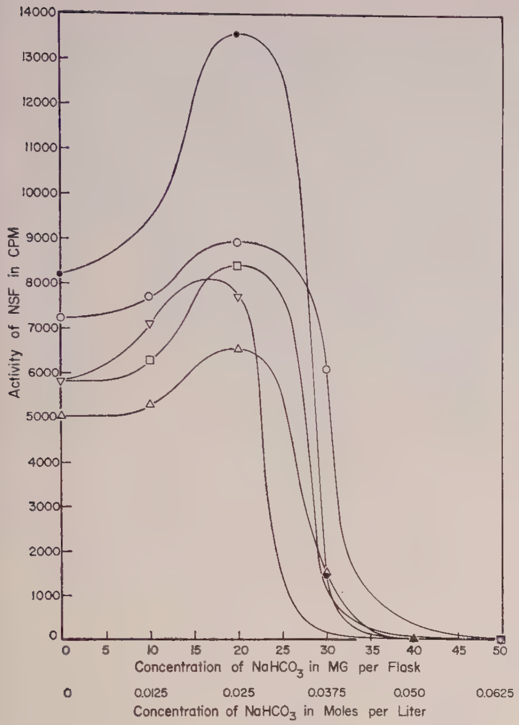

FIG. 1. Biosynthesis of NSF by liver homogenate as influenced by preincubation with various levels of sodium bicarbonate.

served with trypsinized protamine. For this reason, to avoid negative experiments from which no conclusions could be drawn, subsequent experiments have involved either the use of graded levels or an excess of materials to be evaluated against several levels of bicarbonate.

The activity of trypsinized protamine but not undigested protamine against bicarbonate inhibition of the liver system suggested that this activity may be due to arginine which is present in protamine to the extent of 85-90%. The data of Table III, Exp. # 1, show that arginine does indeed reverse bicarbonate inhibition. Some explanation of the figures obtained is in order. As noted earlier, intermediate levels of bicarbonate stimulate the system. Thus if a compound reverses the effect of bicarbonate at intermediate levels of bicarbonate this stimulation should be abolished by the reversing agent. The results obtained with 20 mg of sodium bicarbonate per flask show that this is the case. With no added bicarbonate or arginine (control flask)

TABLE III. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Sodium Bicarbonate and Amino Acids of the Urea Cycle

Exp.	$NaHCO_3$, mg/flask	Additional supplement, mg/flask	NSF, cpm
1			5012
	10		5308
	”	4 Arginine	4653
	”	8 ”	5158
	”	20 ”	4725
	20		6541
	”	4 ”	4915
	”	8 ”	5345
	”	20 ”	5058
	30		1508
	”	4 ”	1840
	”	8 ”	1816
	”	20 ”	6399
	40		35
	”	4 ”	22
	”	8 ”	14
	”	20 ”	106
2			6120
	20		10343
	”	20 Ornithine	7406
	”	” Citrulline	7695
	”	” Arginine	7150
	”	” Canavanine	6853
	30		906
	”	20 Ornithine	8005
	”	” Citrulline	6505
	”	” Arginine	8706
	”	” Canavanine	9885
	40		44
	”	20 Ornithine	291
	”	” Citrulline	29
	”	” Arginine	310
	”	” Canavanine	755

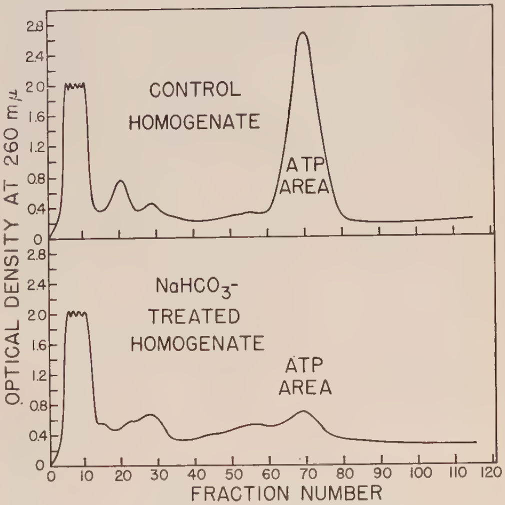

FIG. 2. ATP content of rat liver homogenates preincubated with and without added sodium bicarbonate. Level of sodium bicarbonates employed was 0.05 M.

5012 cpm in NSF were observed. With 20 mg of sodium bicarbonate per flask 6541 cpm in NSF were observed. However, with 20 mg of sodium bicarbonate per flask plus 20 mg of arginine per flask 5058 cpm in NSF were observed. On the other hand, with 30 mg of sodium bicarbonate per flask 1508 cpm in NSF were observed (70% inhibition rather than stimulation). However, with 30 mg of sodium bicarbonate per flask plus 20 mg of arginine per flask 6399 cpm in NSF were observed (essentially complete reversal of bicarbonate inhibition).

The results obtained with arginine suggested that this amino acid reverses bicarbonate inhibition of aerobic activity in essentially whole homogenates of liver by stimulating the Krebs-Henseleit urea cycle. Accordingly a number of amino acids of the cycle as well as canavanine were compared for activity. The data of Table III, Exp. #2 show that all the compounds are active. It is apparent from a study of the results that the order of increasing activity is: citrulline, ornithine, arginine, and canavanine. Thus with no added bicarbonate or amino acid (control flask) 6120 cpm in NSF were observed. With 20 mg of sodium bicarbonate per flask 10343 cpm in NSF were observed. However, with 20 mg of sodium bicarbonate per flask this stimulation was reduced to 7695 cpm with citrulline, to 7406 cpm with ornithine, to 7150 cpm with arginine and to 6853 cpm with canavanine. Again at a level of 30 mg of sodium bicarbonate per flask where inhibition of the conversion of MVA to NSF is 85% complete (6120 cpm in control *vs.* 906 cpm in the sodium bicarbonate flask) 6505 cpm were found in the citrulline flask, 8005 cpm in the ornithine flask, 8706 cpm in the arginine flask and 9885 cpm in the canavanine flask. The results obtained with 40 mg of sodium bicarbonate per flask are compatible with the same order of activity.

Discussion. The results presented demonstrate that bicarbonate is a potent inhibitor of a complete system in aerobic homogenates that is essential for conversion of MVA to NSF. It seems established that bicarbonate is an inhibitor of respiration or of oxidative phosphorylation that is essential for maintenance of ATP levels required for phosphorylation of MVA, phosphorylation of MVA-5-P and decarboxylation and dehydration of MVA-5-PP. These studies do not establish the locus of inhibition but Miller and Evans have shown both with plant and animal preparations that bicarbonate is an inhibitor of cytochrome oxidase(2). Inhibition of cytochrome oxidase by bicarbonate could account for the results presented in this paper. It is perhaps significant that in the two studies bicarbonate showed complete inhibition at 0.05 M. The mechanism by which bicarbonate inhibits cytochrome oxidase has not been established.

It seems clear that the amino acids of the urea cycle reverse bicarbonate inhibition by stimulating the cycle to convert bicarbonate to urea. Reversal of high levels of bicarbonate is apparently not achieved because of limited activity of the cycle. It would appear that citrulline is less active than the other amino acids studied because conversion of citrulline to arginine requires the expenditure of 1 mole of ATP in an ATP limited system without the uptake of bicarbonate. On the other hand, ornithine or arginine (after conversion to ornithine by urease) reduces the level of bicarbonate by 1 mole for every mole of ATP expended. No completely satisfactory mechanism can be offered to explain the fact that canavanine is more active than arginine in reversal of bicarbonate inhibition.

Summary. Bicarbonate is a potent inhibitor of a system essential for maintenance of ATP levels that are required for conversion of mevalonic acid to non-saponifiable material (largely cholesterol) by liver homogenates. This bicarbonate inhibition, over a narrow range of concentration, is reversed by trypsinized protamine or by amino acids of the urea cycle or by canavanine.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Influence of Bacterial Pyrogen on Transplantable Tumors. (26119)

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Crude filtrates from microbial cultures have been shown to be effective oncolytic agents and numerous investigators have observed spontaneous regression of tumors following acute infections. The historical significance affixed to microbes and microbial products in malignancy has been reviewed by other authors(1-4).

During studies in this laboratory on potentiation of the immune response by bacterial pyrogens(5), spontaneously occurring tumors of mice were noted to regress after administration of pyrogen. The work reported here was undertaken to determine the effect of these pyrogens on mouse transplantable tumors. Of the 3 tumors studied, only the subcutaneous transplants of the sarcoma S-37 tumor responded to treatment, as evidenced by tumor regression and a decreased mortality rate.

Materials. The mouse tumors utilized in these experiments were a sarcoma strain S-37, an adenoma strain EO771, and a leukemia strain 1498. The sarcoma S-37 was maintained in this laboratory by serial transplantation in the ABC strain of mice. Likewise the adenoma strain EO771 and the leukemia strain 1498 were both maintained by periodic transplantation in C57BL mice. The ABC and C57BL strains of mice used in the subcutaneous and intraocular homologous transplant experiments were purchased from Roscoe B. Jackson Memorial Cancer Inst., Bar Harbor, Maine. The Swiss albino mice employed in the intracerebral heterologous transplant experiment were obtained from a local source. All animals were allowed free access to their food supplies of Purina Laboratory Chow and water.

The tumor-bearing animals were treated with various doses of the bacterial pyrogenic substances PC-3 and/or PC-6 which were fractions derived from the parent pyrogen PC-1 (Piromen).*

Methods and results. Preliminary work suggested that it was advisable to determine the therapeutic value of pyrogen on tumors at different implant sites as well as on different tumor strains. The effect of these pyrogens on both subcutaneous and intracerebral transplants of the S-37 tumor was ascertained.

The other tumors selected for either subcutaneous or intraocular transplant sites were first removed from a carrier animal and minced with scissors in a small quantity of sterile 0.85% sodium chloride solution. Small fragments of tumor tissue were introduced into the animals by the semi-automatic trocar method of Fuson and Eichwald(6). Subcutaneous tumor transplant of the sarcoma and leukemia strains were implanted into the left axillary region of the recipient mice. Intraocular adenoma transplants were implanted into the anterior chamber of the animal's right eye. For intracerebral transplantation, the trocar method proved inadequate; therefore, the S-37 tumors were removed from the carrier mice and the tumors briefly homogenized in sterile saline with a Waring blendor until the large tissue particles were noticeably disrupted. The Swiss albino mice

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* We are indebted to Dr. Myron Usdin and the Baxter Laboratories, Morton Grove, Ill., for the generous supply of both pyrogenic fractions and lactate buffer.

TABLE I. Influence of Bacterial Pyrogens on Subcutaneous Sarcoma-37 Tumors in ABC Mice.

Group No.*	Fraction inj.	γ per inj. per wk	Total γ inj.	% survival†	P value X^2 test
I	PC-3	10	30	28	.2 –.1
II	PC-6	20	60	42	.05–.02
III	PC-3	10	30	79	.001
	PC-6	20	60		
IV	Control	0	0	0	—

* 14 animals in each group.

† % survival at 90 days with no observable tumor reoccurrence in survivors after 3 weekly injections of bacterial pyrogens.

were then inoculated intracerebrally with 0.03 ml of a 1:200 dilution (w/v) of the saline-tumor suspension. Asceptic technics were employed throughout.

The pyrogenic polysaccharides were dissolved in lactate buffer and the treatment injections administered intraperitoneally in 0.2 ml volumes at the following described time intervals. Animals were inspected daily and both mortality and survival time noted.

Fifty-six ABC mice were inoculated subcutaneously with a small transplant of the tumor S-37. The treatment schedule did not begin until 9 days after tumor implantation, at which time palpable tumors, approximately 7 x 4 mm in size, were observed. The mice were divided into 4 groups of 14 animals each, 7 males and 7 females per group. Group 1 received 3 weekly injections of 10 μg each of fraction PC-3, the second group 3 weekly injections of 20 μg each of fraction PC-6, and the third group 3 weekly injections of a combination of both in the same above amounts. The fourth group of mice served as controls and was accorded similar treatment except that they received 3 weekly injections of a corresponding volume of the pyrogen diluent, lactate buffer. The effect of treatment was dramatic. The per cent survival at 90 days after transplant is presented in Table I. A relationship appeared to exist between amount of drug administered and per cent survival rather than a seemingly higher protective value for one pyrogen as opposed to the other. Total amounts of the administered drugs for the 3 therapy groups were 30, 60, and 90 μg, survival being 28, 42, and 79% respectively. Each of the subcutaneous S-37 tumor transplants responded to pyrogen treatment but only those animals survived in which complete elimination of the tumor occurred. No differences in mortality rate or survival time between males and females were noted. Action upon the tumor resulted in the grossly observable sequence of hemorhage and necrosis followed by occasional sloughing. Tumor growth often reoccurred at marginal areas when the response to the pyrogens was incomplete, and surviving tumor tissue continued to spread until ultimately the animal succumbed. Employing the *Chi* square method of analysis with Yates' correction for small numbers, the dose of PC-3 used failed to increase survival time to a significant degree when compared with those animals receiving the lactate buffer. Administration of the PC-6 and the combination PC-3 plus PC-6 resulted in significant protection against the S-37 tumor.

Two hundred and fifty albino mice were inoculated intracerebrally with the S-37 tumor. The tumor-bearing animals were then randomly subdivided into 4 major groups of 25 animals which received no therapy and 3 groups of 75 animals which received treatment either on the first, third, or fifth day following tumor transplantation. Each major group of 75 animals was further divided into 3 sub-groups A, B, and C, for treatment with PC-3, PC-6, or a combination of both as outlined in Table II. Dosages used were the same as those for the subcutaneous S-37 transplantation experiment. Pyrogen administration followed at 4-day intervals from the initial treatment doses. The slight differences in survival time between control animals and those treated with the pyrogen fractions may be seen in Table II. It should be noted, however, that there were no surviving animals. These results show that mice challenged *via* the intracerebral route with the S-37 tumor did not exhibit an increased survival time when treated with these two bacterial pyrogens, regardless of dosage employed or therapeutic time interval.

Adenoma EO771 was implanted intraocularly into sixty C57BL mice and the mice randomly separated into groups of 20 animals each. Groups I, II, and III received a mix-

TABLE II. Effect of Bacterial Pyrogens upon Intracerebral Transplants of Tumor S-37.

Group No.*	Pyrogen fractions/inj.	Time of 1st treatment after transplant, days	Avg survival time, days
I	A 10 µg PC-3	1	12.52
	B 20 " PC-6	1	12.52
	C 10 " PC-3 20 " PC-6	1	13.04
II	A 10 " PC-3	3	12.76
	B 20 " PC-6	3	12.12
	C 10 " PC-3 20 " PC-6	3	13.48
III	A 10 " PC-3	5	13.32
	B 20 " PC-6	5	13.16
	C 10 " PC-3 20 " PC-6	5	13.20
IV	Normal controls	—	13.40

* 25 animals in each group.

ture of 20 µg of PC-6 plus 10 µg of PC-3, a mixture of 10 µg of PC-6 plus 5 µg of PC-3, or a corresponding volume of the lactate buffer, respectively. Four weekly injections of either the pyrogenic fractions or lactate buffer were administered beginning 10 days after tumor implantation. The results are given in Table III. Under the described experimental conditions, the adenoma EO771 also failed to respond to pyrogen treatment. Average survival time for each treated group was approximately that of the control group.

Leukemia 1498 was transplanted subcutaneously into one hundred C57BL mice and the mice randomly subdivided into 4 groups of 25 animals each. Pyrogen administration was initiated at 2 different time intervals and in 2 dosages beginning 48 hours after implantation. The first group of 25 animals received an intraperitoneal injection of 20 µg of fraction PC-6 and 10 µg of fraction PC-3 every 4 days. The second group of 25 animals was accorded the same dosage except that treatment was given every 2 days. Group III received an intraperitoneal injection of 10 µg of PC-6 plus 5 µg of PC-3 every 2 days. The remaining 25 animals constituted Group IV, the tumor controls, and received no therapy. It is apparent from the results (Table IV) that leukemia 1498 did not respond to the pyrogen treatment. Average survival time of the treated animals was slightly less than that of control animals.

TABLE III. Effect of Bacterial Pyrogens on Intraocular Transplanted Tumor EO771 in C57BL Mice.

Group No.*	Pyrogen fraction per inj.	Avg survival, days
I	20 µg PC-6 10 " PC-3	43.2
II	10 " PC-6 5 " PC-3	49.7
III	0	48.0

* 20 animals in each group with each animal receiving 4 weekly injections.

TABLE IV. Effect of Bacterial Pyrogens on Transplanted Leukemia 1498 in C57BL Mice.

Group No.*	Treatment interval, days	Pyrogen fraction/inj.	No. of inj.	Avg survival, days
I	4	20 µg PC-6 10 " PC-3	2	12.7
II	2	20 " PC-6 10 " PC-3	3	12.1
III	2	10 " PC-6 5 " PC-3	3	12.6
IV	Control	0	0	13.2

* 25 animals in each group.

Discussion. Naturally occurring mouse tumors had been noted to undergo regression following administration of a pyrogenic-vaccine combination. This result was attributed to the 2 pyrogens contained in the vaccine and both were tested for their antineoplastic activity on transplantable mouse tumors. These data indicate that only the subcutaneous transplants of the sarcoma S-37 tumors were amenable to pyrogen therapy. Sarcoma S-37 tumors that were not completely eliminated during treatment often became refractory to further treatment and ultimately progressed to kill the animal. Refractoriness to pyrogens has been commonly observed. Animals readily become incapable of eliciting a marked febrile response after repeated pyrogen administration (7-9). The tolerance to pyrogenic bacterial materials has been explained by both humoral inhibitors (10) and accelerated elimination by the reticuloendothelial system(11). Tumor destruction apparently occurred through a vascular response similar to either the hypotensive response of Algire(12) or to the Shwartzman reaction as previously ascribed to other bacterial-derived antineoplastic agents(13-14). Likewise, a refractive state may also be induced toward both the Shwartzman reaction(15-17) and the hypotensive state(12).

Summary. Of the 3 tumor sites tested, only the subcutaneous transplants of the S-37 tumors responded to treatment with the 2 pyrogenic fractions PC-3 and PC-6. Regardless of the treatment regimen, the intracerebral S-37 transplants failed to respond to treatment with pyrogen. Animals bearing either the intraocular transplants of adenoma EO771 or the subcutaneous transplants of leukemia 1498 exhibited no increase in survival time as a result of pyrogen therapy.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Insulin Antagonism in Serum of Untreated Diabetics and in Previously Treated Diabetics with Ketoacidosis.* (26120)

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It has already been shown that sera of uncontrolled diabetics, who have previously been treated with insulin, neutralize the activity of insulin when tested *in vitro* by the rat diaphragm assay(1). This antagonism disappears when the diabetes is brought under control. As far as we know, no one has tested the effect of added insulin to sera from new and untreated diabetics, where no insulin antibodies are present. The present communication describes studies in which the biological effect of insulin on the rat diaphragm incubated in sera of new and untreated diabetics is compared to its effect when incubated in sera of patients with ketoacidosis, who had been previously treated with insulin.

Materials and methods. Blood was obtained from: 1) recently discovered diabetics with an elevated blood sugar and glycosuria; these patients never received insulin, and 2) patients admitted to the hospital with untreated diabetic acidosis; although they had had no insulin treatment for many hours prior to admission, they all had received insulin for over 2 months in the past. The serum was removed after proper blood clotting and kept at –10°C until time of testing. Sera with a glucose content grossly over 300 mg% were dialysed against a bicarbonate buffer(2) to decrease the glucose content to this approximate range. The same buffer, with glucose added (300 mg%), was used for the bioassay. The specimens were assayed for insulin or

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* Supported by U.S.P.H.S. grant.

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TABLE I. Untreated New Diabetics.

		Buffer		Serum			
Case No.	Age	Insulin added (μU/cc)	Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)	Insulin added (μU/cc)	Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)	% inhibition of added insulin	Blood sugar
10	41	1000	246 ± 28	0	0 ± 13		200
”	”	”	”	1000	168 ± 2	70	”
11	40	1000	210 ± 23	0	4 ± 8		250
”	”	”	”	1000	116 ± 13	84	”
12	58	1000	328 ± 8	0	166 ± 15		380
”	”	”	”	1000	312 ± 30	14	”
13	50	1000	280 ± 18	1000	144 ± 8	85	374
14	58	500	300 ± 13	0	196 ± 10		288
”	”	”	”	500	258 ± 15	37	”
15	56	500	162 ± 10	0	294 ± 15		392
”	”	”	76 ± 2	500	352 ± 32	0	”
16	73	500	266 ± 25	500	46 ± 40	94	382
17	42	500	214 ± 15	500	70 ± 15	68	300
18	79	500	214 ± 15	500	94 ± 22	90	360
19	39	500	314 ± 12	500	162 ± 28	87	380
20	30	500	336 ± 23	500	292 ± 13	27	480
21	65	500	270 ± 15	500	30 ± 10	95	392
22	31	500	228 ± 17	500	148 ± 32	75	585
23	33	500	230 ± 17	500	96 ± 2	88	200
24	30	500	138 ± 22	500	90 ± 10	72	278
25	51	500	274 ± 42	500	212 ± 23	47	281

anti-insulin activity by the rat diaphragm method of Vallance-Owen *et al.*(3), using the intact serum. In each experiment the glucose uptake by serum with added insulin[‡] was compared with the uptake in buffer alone (basal uptake) and buffer with added insulin. When enough material was available, inherent serum insulin activity was also tested. Each category was determined in triplicate by use of hemidiaphragms from 3 different rats.

One acidotic serum and sera of 2 normal subjects were subjected to hanging curtain electrophoresis(4), using a barbital buffer, pH 8.6, ionic strength 0.05. Position of the conventional protein fractions was determined by staining the curtains with ninhydrin. The collected fractions were dialysed against 20% Plasdone C[§] overnight to remove the buffer and most of the water, then reconstituted to original serum volume with bicarbonate buffer for insulin bioassay. All fractions were frozen until time of assay, and glucose was then added to make the concentration 300 mg%. Inadequate amounts of serum prevented us from testing these specimens prior to electrophoresis.

Results and discussion. Our series consists of sera from 25 diabetics: 16 with ketoacidosis and no treatment and 9 with ketoacidosis and previous insulin treatment. Table I shows that the sera of most of the new diabetics markedly blocks the action of added insulin, although in a few cases (Nos. 12, 14, 15, and 20) the loss of activity is not significant. One patient, (Case #15), showed levels above 1000 μU/cc both with and without added insulin. Since the difference in glucose uptake between 500 and 1000 microunits is relatively small, the difference from day to day in the bioassay results would not become apparent unless both concentrations were tested simultaneously. Table II gives the data obtained in diabetics with ketoacidosis. Degree of inhibition does not seem to vary much from that of the new diabetics. There seemed to be little correlation with blood sugar, CO_2, or lipemia. Case No. 2, an elderly woman who died in shock 3 hours after admission, showed very little inhibition of

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[‡] Purified insulin supplied by Dr. O. K. Behrens, Eli Lilly Co., Indianapolis, Ind.

[§] Grade of polyvinylpyrrolidone, Antara Chemicals, New York City.

TABLE II. Diabetic Ketoacidosis.

		Buffer		Serum					
Case No.	Age	Insulin added (μU/cc)	Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)	Insulin added (μU/cc)	Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)	% inhibition of added insulin	Blood sugar	CO_2	Lipemia
1	35	1000	200 ± 23	0	74 ± 22		298	—	No
	”	”	”	1000	72 ± 10	90	”	—	”
2	74	1000	254 ± 27	1000	218 ± 10	29	1400	5.0	No
3	20	500	258 ± 15	500	212 ± 7	40	456	8.0	Yes
4	23	500	258 ± 15	500	160 ± 15	74	550	—	”
5	21	500	214 ± 15	500	72 ± 7	95	672	—	”
6	22	500	266 ± 25	500	100 ± 7	94	348	10.0	No
7	24	500	162 ± 10	500	12 ± 22	97	456	4.8	Yes
8	30	500	228 ± 17	500	120 ± 22	86	832	5.7	”
9	25	500	230 ± 17	500	154 ± 7	68	812	7.0	Lip. ret.*

* Lipemia retinalis.

the added insulin. All the others were typical juvenile-type diabetics with acetonemia and clinical symptoms of ketoacidosis at time of admission. Case No. 9 had a relatively low blood sugar, mild acetonemia, and relatively mild symptoms. All of these cases needed less than 1000 units/24 hours for ultimate control of the condition. Case No. 2 had received 300 units at time of death. Table III shows data obtained on 5 normal sera. No gross antagonism can be seen.

TABLE III. Normal Sera without and with Addition of Insulin (500 μU/cc) Compared with Buffer + Insulin (500 μU/cc).

		Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)		
Case No.	Age	Buffer+ insulin	Serum alone	Serum + insulin
1	26	266 ± 34	134 ± 22	322 ± 14
2	25	162 ± 26	156 ± 16	196 ± 15
3	31	280 ± 43	164 ± 17	262 ± 14
4	22	252 ± 45	152 ± 25	288 ± 13
5	25	264 ± 11	68 ± 8	260 ± 44

Table IV shows bioassay data obtained on one acidotic serum after hanging curtain electrophoresis. Since several fractions showed no antagonism to insulin added, especially in the γ and α region, they were assayed without addition of insulin. High insulin activity was found in fractions 4 (166 μU/cc), 5 (570 μU/cc), 7, and 8 (710 μU/cc) when plotted on the curve of the concurrent insulin standard. Table V shows data obtained on 2 normal sera subjected to hanging curtain electrophoresis. The total amount of insulin activity is far less than that of the patient in ketoacidosis. These findings agree closely with those of Randle and Taylor(5,6,7). The reported insulin antagonist of Field and Stetten(8) was not found, using our technic.

We feel that these data suggest the presence of substances in the blood of new diabetics, as well as diabetics in ketoacidosis which can combine with insulin to neutralize its effect on glucose uptake by the rat diaphragm. In the new diabetics this is presumably not an antibody. Recent work(9) indicates that insulin-basic protein complexes present in normal sera do not produce an insulin-like effect on glucose uptake of the rat diaphragm, while they do stimulate glucose uptake by the rat adipose tissue. Precipitation of the basic protein(s) at pH 10, or preincubation with rat adipose tissue extract (10), restores the insulin-like effect on the rat diaphragm. From the electrophoresis experiment it appears that a large quantity of insulin is similarly liberated by this technic. It is possible that new diabetics, (and diabetics in acidosis) possess excessive amounts

TABLE IV. Diabetic Ketoacidosis: Serum Insulin Activity after Hanging Curtain Electrophoresis.

		Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)				
Fraction No.	Protein band location*	Buffer + insulin (1000 μU/cc)	Fraction + insulin (1000 μU/cc)	Buffer + insulin (1000 μU/cc)	Fraction alone	Insulin activity (μU/cc original serum)
2				202 ± 7	−30 ± 23	0
3				238 ± 7	−58 ± 7	0
4		204 ± 13	232 ± 33		152 ± 15	160
5	γ			238 ± 7	198 ± 18	570
6				202 ± 7	76 ± 22	40
7	β			332 ± 30	122 ± 8	50
8		204 ± 13	236 ± 27	178 ± 11	162 ± 2	710
9	α	222 ± 13	204 ± 17	200 ± 33	44 ± 22	<10
10		186 ± 10	228 ± 8	178 ± 11	84 ± 23	90
11		204 ± 13	226 ± 8			
12	alb.	186 ± 10	206 ± 32			
13		222 ± 13	220 ± 8			
14						
15	pre-alb.			268 ± 35	−26 ± 43	0

* Identification by acid ninhydrin stain of curtain.

TABLE V. Normal Sera Insulin Activity after Hanging Curtain Electrophoresis.

		Glucose uptake above basal level ± S.E. (μg/10 mg dry diaphragm)					
		Subject R.M.			Subject D.S.		
Fraction No.	Protein band location	Buffer and insulin*	Serum fraction	Insulin activity†	Buffer and insulin*	Serum fraction	Insulin activity†
4		294 ± 58	26 ± 7	<10			
5	γ	308 ± 9	62 ± 24	<10	234 ± 27	144 ± 22	216
6		210 ± 4	10 ± 33	<10	234 ± 27	54 ± 17	15
7	β	240 ± 19	1 ± 11	<10	242 ± 24	38 ± 14	<10
8		240 ± 19	6 ± 11	<10	242 ± 24	62 ± 7	14
9	α	210 ± 4	32 ± 10	<10	326 ± 17	40 ± 23	<10
10		240 ± 19	4 ± 6	<10	326 ± 17	92 ± 13	22
11		210 ± 4	2 ± 7	<10	320 ± 7	50 ± 6	<10
12	alb.	254 ± 28	122 ± 13	100	320 ± 7	0 ± 9	0
13		210 ± 4	−28 ± 18	0			

* 1000 μU/cc. † μU/cc original serum.

of basic proteins in their sera which bind most of the insulin released from the pancreas, thus reducing the amount of free insulin available to certain tissues. Thus, the onset of diabetes may not result from underproduction of insulin, but rather from inactivation by excess binding.

Summary. Undiluted sera of new, untreated diabetics and diabetics in ketoacidosis have been shown to exhibit antagonism to added insulin when assayed by the rat diaphragm technic. One acidotic serum showed unusually high insulin activity after hanging curtain electrophoresis. The possible significance of these data is discussed.

We wish to thank Miss Ruth Miller for invaluable technical assistance in this work, and Drs. Joseph Crampton and Robert Reeves, Virginia Mason Hosp. for aid in obtaining suitable sera.

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Received Sept. 6, 1960. P.S.E.B.M., 1960, v105.

Effect of Aldosterone Antagonist on *in vitro* Production of Aldosterone and Corticosterone in Rats.* (26121)

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Several investigators(1-3) have demonstrated that spirolactones (*e.g.*, SC-8109), are antagonistic to the electrolyte-regulating effects of aldosterone at the level of the kidney tubule. It has been suggested that the spirolactones influence the pattern of renal electrolyte excretion particularly in conditions where the level of aldosterone is elevated(4,5). Agents such as amphenone inhibit aldosterone production as well as other corticoids by suppressing adrenal gland secretion(6). The spirolactones while competing with aldosterone at peripheral sites *i.e.*, the kidney tubule, do not have a direct effect upon the adrenal gland. In fact, the spirolactones have been shown to cause increased adrenal gland production of aldosterone *in vivo*(7,8). We have found increased *in vitro* production of aldosterone in adrenals taken from rats previously treated with the spirolactone, SC-8109.

Materials and methods. Young adult virgin male(24) and female(24) Sprague-Dawley rats averaging 210 ± 15 and 160 ± 15 g of body weight respectively were used. Twelve males and 12 females were injected with a sesame oil solution of spirolactone, SC-8109,[†] 10 mg/ml. Each animal received 1.5 mg/100 g of body weight, subcutaneously, once every Mon., Wed., and Fri. for 12 weeks. Control males (9 rats) and females (12 rats) were given the sesame oil vehicle (0.25 ml/100 g b.w.) on the same regimen of injection as the spirolactone. The animals were housed in air-conditioned quarters and fed a regular diet with tap water to drink *ad libitum*. At autopsy, animals were sacrificed by decapitation. Their adrenal glands were removed and trimmed free of extraneous tissue as rapidly as possible. Adrenals of 3 animals were halved and pooled for each incubation. The incubation procedure and method for extracting the steroids have been described(9). ACTH (Acton "X") in a concentration of 100 $m\mu/100$ mg adrenal tissue was present in the incubation medium. The incubation technic was adapted from Saffran and Schally(10). The glands were preincubated for 30 min. Fresh incubation medium was added and the incubation continued for 1 hour. Total alpha-ketolic steroids in the final incubate were determined by the blue tetrazolium method of Nowaczynski *et al.*(11). Quantitative paper chromatography was used to separate the steroids. Individual steroids were determined directly by scanning the chromatograms with a recording and integrating densitometer (12). Changes in production of aldosterone and corticosterone relative to total alpha-ketolic steroids are reported here.

Results. No unusual changes occurred in the behavior of the animals during the course of injections. The SC-8109-treated males gained less weight than the sesame oil-injected male controls. The opposite trend was observed in the female rats (Table I). Adrenal weight of SC-8109-treated females was significantly less than the female controls ($p<0.03$).

A striking increase in relative production of aldosterone *in vitro* was found in SC-8109-treated incubated adrenals of male rats (Table I). This was accompanied by a significant reduction of corticosterone production. Female rats treated with SC-8109 also showed an increase in aldosterone with a concomitant fall in corticosterone. However, this was not as marked as in the male.

Discussion. The significant increase in aldosterone production ($p<0.001$) in male

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* This work was supported in part by the John A. Hartford Foundation, Gustavus & Louise Pfeiffer Foundation, Am. Heart Assn., and U.S.P.H.S.

[†] SC-8109 was kindly provided by Dr. Victor A. Drill, G. D. Searle Co., Skokie, Ill.

TABLE I. *In Vitro* Adrenal Production of Aldosterone and Corticosterone in Male and Female Sprague-Dawley Rats Treated with Spirolactone (SC-8109).

Group	No. rats	Body wt, g	Adrenal wt, mg	Total steroid, µg/hr/100 mg adrenal tissue	Aldosterone	Corticosterone
					% of total steroid	
♂						
Sesame oil	9	394 ±17*	45.8 ± 1.9	11.2 ± .1	17.5 ± 2.1	52.6 ± 3.0
SC-8109/sesame oil	12	363 ± 8	42.0 ± 2.3	12.1 ±1.0	37.4 ± 1.5 P <.001	33.4 ± 1.6 P <.005
♀						
Sesame oil	12	242 ± 6	65.2 ± 2.7	13.4 ± .5	13.9 ± 2.4	55.2 ± 2.4
SC-8109/sesame oil	12	260 ± 6	57.4 ± .5 P <.03	14.4 ± .5	23.1 ± 2.5 P <.05	41.4 ± 4.1 P <.05

* Stand. dev. of mean.

rats treated with SC-8109 over sesame oil-injected controls confirms what has been observed *in vivo*(7,8). The greater production of aldosterone by SC-8109-treated male adrenals compared to similarly treated females may reflect in part the greater capacity of male Sprague-Dawley rats to produce aldosterone (*in vitro*)(12). The larger renal tubular mass in males provides more opportunity for competition between aldosterone and SC-8109. This may result in increased adrenal production of aldosterone by way of feed-back mechanisms.

The significant reduction of corticosterone observed in both sexes seems to parallel the increase in aldosterone, and probably reflects changes in adrenal steroidogenic pathways which favor aldosterone production.

Summary. Male and female Sprague-Dawley rats were treated with the spirolactone, SC-8109, known to be an aldosterone antagonist. After 12 weeks of treatment the animals were sacrificed. Adrenal glands were incubated *in vitro* and total alpha-ketolic, as well as individual, steroids were determined. Male and female adrenal incubates from SC-8109-treated rats contained significantly more aldosterone and less corticosterone than incubates from those injected with the sesame oil vehicle. Male rats treated with SC-8109 showed particularly striking increase in aldosterone production.

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Received Sept. 6, 1960. P.S.E.B.M., 1960, v105.

Pathogenicity and Immunogenicity Herpes Simplex Virus Strains Propagated in Rabbit Kidney Tissue.* (26122)

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Cultivation of herpes simplex virus (HSV) in tissue culture of rabbit kidney (RK) has been reported(1,2,3). In this laboratory application of this technic has made possible a quantitative study of the pathogenicity and immunogenicity of strains of HSV. Results with strains of HSV with different passage histories will be compared.

Materials and methods. HSV strains: The HF strain used was first described by Flexner and Amoss(4) and has undergone numerous passages in rabbits, mice and chick embryos. The strain employed in this study (HF 378) was adapted to grow in the allantoic cavity of embryonated eggs. Infected allantoic fluid possessed 10^7 or more LD_{50} for 7-day-old chick embryos *via* yolk sac inoculation. Strain 1166,† a chick embryo adapted virus was originally isolated by Armstrong in mice from the spinal fluid of a case of aseptic meningitis (5). Strain 1266‡ was isolated in suckling mice from the oral lesion of a patient with aphthous stomatitis(6). Strain L4‡ was isolated from a labial vesicle and passed in chick embryo tissue culture. All strains were readily adapted to grow in RK cultures. For the present study, virus pools of the 8th RK passage of strain HF, 4th RK passage of strain 1166, 18th RK passage of strain 1266 and 5th RK passage of strain L4 were prepared. Virus titers in RK cultures were $10^{9.0}$, $10^{8.5}$, $10^{9.2}$ and $10^{9.0}$ $TCID_{50}$/ml respectively.

Cultivation and titration. Kidneys of young rabbits weighing 2 to 3 lb were trypsinized according to the technic of Youngner (7). The growth medium consisted of 0.5% lactalbumin hydrolyzate in Earle's balanced salt solution with addition of 0.01 M Tris (hydroxymethyl aminomethane), 5 - 10% horse serum and 100 μg of dihydrostreptomycin and 100 units of penicillin G or polymyxin B per ml. After complete sheets of epithelial cells had formed, the virus was propagated in Parker medium 199 without serum and cell degeneration was complete in approximately 40 hours. Fifty percent end point was calculated according to method of Reed and Muench(8). Virus titers were expressed as number (logs) of infectious or lethal doses per ml. By calculating the ratio between tissue culture titer and egg or mouse titer of the same virus fluid, the number of $TCID_{50}$ required to produce 1 LD_{50} was estimated.

Immunization of animals. Virus was inactivated at 37°C for 24 hours by adding formalin to the tissue culture fluid to a final concentration of 1:4000. For immunization of guinea pigs, 3 doses of 1 ml each were given i.p. at weekly intervals. In mouse protection experiments, mice were immunized intraperitoneally at weekly intervals with 0.5 ml of vaccine for 3 injections. Guinea pigs were bled one week post-immunization, the sera inactivated at 56°C for 30 minutes, pooled and stored at –20°C until used.

Results. Pathogenicity of HSV. A. *RK Cells.* Three types of cytopathogenic effect were distinguishable. With strain HF, the chief feature consisted of giant cell formation, whereas strain 1166 produced a clumping or fusing together of cells. The characteristic CPE of strains 1266 and L4 was rounding and "ballooning" of the cells and aggregates generally formed. The CPE characteristic for each strain remained constant through repeated passages in RK culture. B. *Chick embryos.* All 4 strains produced typical pocks on the chorioallantoic membrane. Irrespective of their previous passage history, all were lethal for the 7-day chick embryo upon yolk sac inoculation. However, strains differed in

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* Preliminary report presented at Soc. Am. Bact., Philadelphia, May 1960, (*Bact. Proc.*, 1960, 124).

† This virus was kindly supplied by the Virus Lab., Univ. of Michigan where it had been adapted to grow in the chick embryo.

‡ The authors are indebted to Dr. Sam C. Wong, Cornell Univ. Med. Coll. for this virus.

TABLE I. Pathogenicity of HSV for Chick Embryos and Mice.

Strain	TC passage	TCID$_{50}$/ml	Chick embryo (7 day) LD$_{50}$/ml	TCID$_{50}$/LD$_{50}$	Mouse (4 wk) LD$_{50}$/ml	TCID$_{50}$/LD$_{50}$
HF	8	$10^{9.0}$	$10^{7.6}$	25	$10^{6.0}$	1,000
1166	4	$10^{8.5}$	$10^{5.9}$	500	$<10^{1.5}$	$>$10,000,000
1266	18	$10^{9.2}$	$10^{5.6}$	4,000	$10^{6.5}$	500
L4	5	$10^{9.0}$	$10^{5.1}$	7,900	$10^{6.1}$	790

virulence as indicated by the number of TCID$_{50}$ required to kill the embryo (Table I). C. *Mice.* With the exception of strain 1166, the other 3 strains were highly pathogenic for the mouse *via* the intracerebral route (Table I). Several hundred to a thousand TCID$_{50}$ were required to infect a 4-week-old mouse by intracerebral inoculation. This dosage remained at this level through repeated passages in RK culture (Table II). On the other hand, strain 1166, which had been shown to possess low pathogenicity for the mouse brain after passages in eggs(9), failed to produce infection following injection of 10^7 TICD$_{50}$ of virus. However, virulence for the mouse was not entirely lost, because the virus was still lethal for suckling mice or adult mice treated with cortisone (5 mg per mouse).

TABLE II. Effect of Passages in RK Culture on Pathogenicity of HSV for Mouse Brain.

Strain	Culture passage	LD$_{50}$/ml	TCID$_{50}$/ml	TCID$_{50}$/LD$_{50}$
1266	3	$10^{6.2}$	$10^{8.5}$	200
	8	$10^{5.9}$	$10^{8.3}$	250
	12	$10^{5.5}$	$10^{8.0}$	320
	18	$10^{6.5}$	$10^{9.2}$	500
1166	1	$10^{3.0}$	$10^{7.0}$	10,000
	2	$10^{2.0}$	$10^{8.3}$	2,000,000
	4	$<10^{1.5}$	$10^{8.5}$	$>$10,000,000

The mouse can also be infected through various routes. The threshold of susceptibility varied widely depending on route of inoculation. Contrary to expectation, intravenous inoculation was the least effective. In any case, CNS involvement appeared to be the principal cause of death (Table III). That susceptibility of the mouse to HSV was inversely related to age(10) was clearly confirmed by present results (Table IV). This was evident regardless of route of inoculation. The newborn mouse appeared to be equally or slightly more sensitive than RK cells for detection of HSV.

TABLE III. Susceptibility of 4-Wk-Old Mice to Strain 1266 via Various Routes of Inoculation.

Route of inoc.	LD$_{50}$/ml	TCID$_{50}$/ml	TCID$_{50}$/LD$_{50}$
I.C.	$10^{6.5}$	$10^{9.2}$	500
I.P.	$10^{4.6}$	$10^{9.2}$	40,000
I.M.	$10^{4.2}$	$10^{9.2}$	100,000
I.N.	$10^{3.5}$	$10^{9.2}$	500,000
I.V.	$10^{2.4}$	$10^{9.2}$	6,300,000

Pathogenicity of HSV for other Laboratory Animals. Table V shows differences in susceptibility of other laboratory animals to the 4 strains of HSV inoculated intracerebrally. Undiluted virus fluid was used and the inoculum contained 10^7 or more TCID$_{50}$. Adult hamsters and cotton rats were susceptible to all 4 strains. Although the strains were virulent for suckling rabbits and suckling white rats they failed to induce infection in adult animals. Guinea pigs appeared to be refractory to i.c. infection. However, all strains produced severe corneal and dermal lesions in the rabbit or foot-pad lesions in the guinea pig when inoculated accordingly. Pathogenicity of HSV for the CNS of monkeys would appear to depend on virus strain as well as species of monkey. For example, strain HF was non-pathogenic for both cynomolgus and rhesus monkeys; strain 1266 infected only cynomolgus but not rhesus; while strain 1166 was virulent for both species. The lesion produced was mainly a localized meningitis with variable amount of parenchymal involvement. The ganglion cells were only

TABLE IV. Influence of Age on Susceptibility of Mice to Strain 1266 via I.C. and I.N. Inoculations.

Route	Age of animal	LD$_{50}$/ml	TCID$_{50}$/ml	TCID$_{50}$/LD$_{50}$
I.C.	1 day	$10^{9.9}$	$10^{9.2}$	.2
	4 wk	$10^{6.5}$	$10^{9.2}$	500
	8 "	$10^{4.7}$	$10^{9.2}$	32,000
I.N.	3 days	$10^{5.0}$	$10^{9.2}$	16,000
	4 wk	$10^{3.5}$	$10^{9.2}$	500,000

TABLE V. Pathogenicity of Strains of HSV for Various Animal Species via I.C. Inoculation.

	Rabbit		Guinea pig		Albino rat			Cotton	
Strain	Adult	Suckling	Adult	Suckling	Adult	Suckling	Hamster	rat	Monkey
HF	0/4*	2/3	0/3	0/3	0/3	1/3	2/3	1/2	0/2
1166	0/4	1/3	0/3	1/3	0/3	2/3	1/3	1/3	4/4
1266	0/4	3/3	0/3	0/3	0/3	2/3	3/3	3/3	1/4†
L4	0/4	3/3	0/3	0/2	0/3	1/3	2/3	2/3	ND

* No. died/No. inoculated. † One cynomolgus monkey was infected but did not die.

ND = Not done.

TABLE VI. Immunogenic Potency of Formalin-Inactivated HSV (Strain 1266) Grown in RK Culture.

A. Antibody production in guinea pigs

Immunization	Serum neutralization* titer†
None	<10
1 dose	80
3 doses	640

B. Immunization of mice against I.C. homologous challenge

Immunization	Virus dilutions 10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer	Protection index
None		5/5‡	4/5	5/5	3/5	0/5	0/5	$10^{-4.0}$	—
1 dose	2/5	3/5	0/5	1/5	0/5	0/5		$10^{-0.7}$	3,000§
2 doses	3/5	0/5	1/5	0/5	0/5	0/5		$10^{-0.3}$	5,000
3 ”	0/4	1/5	0/5	1/5	0/5	0/5		$<10^0$	>10,000

* *Vs* 100 $TCID_{50}$ in RK tubes.
† Titer expressed as reciprocal of serum dilution.
‡ Mice died/mice inoculated.
§ No. of LD_{50} resisted.

slightly affected or not at all and virus could be recovered from the infected nervous tissue.

Immunogenicity of HSV. Study of immunogenic potency was limited to production of neutralizing and protective antibodies in guinea pigs and mice. However, it should be pointed out that antiserum against strain 1166 failed to neutralize the other 3 strains and *vice versa,* whereas no antigenic difference was noted among the other strains. Neutralization tests were carried out in tube cultures of RK cells. Antiserum against B virus§ also did not neutralize strain 1166.

TABLE VII. Comparison of Immunogenicity of Formalin-Inactivated HSV Propagated in Egg, Mouse Brain, and RK Cultures.

		Guinea pigs	Mice
Strain	Virus preparation*	Serum neutralization titer*	Protection index†
HF	Allantoic fluid	40	10
	RK culture ”	320	>10,000
1266	Mouse brain suspension	640	>32,000
	RK culture fluid	320	25,000

* *Vs* 100 $TCID_{50}$ in RK tubes; titer as reciprocal of serum dilution.
† No. of LD_{50} resisted.

Results of an evaluation of immunogenic potency of formalin-inactivated RK grown virus (strain 1266) are presented in Table VI. Virus titer of the fluid before inactivation was $10^{8.5}$ $TCID_{50}$/ml. Immunogenic potency of preparations of HSV grown in RK culture, embryonated eggs and suckling mouse brain were compared by employing similar evaluation procedures. In this experiment, virus titers of the preparations were adjusted to $10^{8.0}$ $TCID_{50}$/ml before inactivation by formalin. Results indicated that RK culture virus and mouse brain virus were antigenically comparable, while the chick embryo propagated virus appeared to be much less so both in antibody production in guinea pigs as well as in mouse protection tests (Table VII).

Discussion. It is well recognized that strains of HSV vary with respect to pathogenicity for different hosts and tissues. Moreover, HSV manifests varying types of cytopathogenicity in tissue culture systems(11, 12). Difference in cytopathogenicity would appear to suggest heterogeneity of the virus population and by laboratory manipulation strains could be obtained which showed predominance of certain types of response(12). A similar explanation may be applied to differences in pathogenicity for various animal species. It is conceivable that passage of the virus in certain host animal or tissue may well serve as a selective process for virus particles with the specific attribute.

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§ The authors are grateful to Dr. K. Hummeler, Childrens Hosp., Philadelphia, Pa. for this serum.

It is generally held that very little antigenic variation exists among strains of herpes simplex virus. In this regard, it is of interest that strain 1166 which came to us after 31 egg passages showed little or no antigenic relationship to the other strains. This finding, however, agrees with the results of Florman and Trader(13) who found that the mouse-passage virus of the Armstrong strain was antigenically unrelated to other herpes strains in cross neutralization tests in mice. Since these observations were at variance with those in the original report(5) the matter merits further investigation.

Except for the results reported for strain 1166, the other strains grown in RK culture were highly pathogenic for the mouse and the chick embryo irrespective of their previous contact with these hosts. In contrast to their virulence for the mouse CNS, the same virus failed to induce disease in the rabbit brain, although a much larger dose of infectious virus was inoculated.

Our results indicate that HSV propagated in RK cultures is highly antigenic even after formalin inactivation. The finding that virus grown in eggs is much less potent in protecting mice against intracerebral challenge agrees with the observation of Jawetz and associates(14). As tissue culture grown virus can be prepared relatively free from extraneous proteins, such preparation may be more suitable for immunization studies in man.

Summary. Four strains of HSV have been propagated in high titer in RK monolayer culture. Marked strain differences exist with regard to cytopathogenic effect as well as virulence for chick embryos, mice and other animals. Tissue culture propagated HSV is much more highly antigenic than the same virus strain grown in the chick embryo.

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Received Sept. 15, 1960. P.S.E.B.M., 1960, v105.

Chlorotetracycline and Growth Promoting Effect of Beta Carotene and Vitamin A in Rats.* (26123)

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Some of the antibiotics have been found effective in producing additional growth in experimental animals, particularly those experimental subjects maintained under unsanitary conditions and subsisting on diets deficient in some of the water-soluble vitamins. This increased growth rate has been attributed to: (a) partial elimination of the microorganisms from the digestive tract, (b) increased vitamin synthesis in the digestive tract, (c) improved absorption of vitamins and other nutrients, and (d) other factors.

More recent reports indicate that some of the antibiotics when incorporated in the ration also stimulate additional growth in Vit. A-deficient animals and increase liver storage of the vitamin. Almquist *et al.*(1), Burgess *et al.*(2,3) and Coates *et al.*(4) have reported that penicillin and/or chlorotetracycline feeding improved utilization of it. A and/or carotene by the growing chick as indicated by blood level and liver storage of the vitamin. Barber *et al.*(5) reported increased hepatic Vit. A following chlorotetracycline feeding but not following the feeding of penicillin. High(6) reported that chlorotetracycline feeding increased liver and kidney storage of Vit. A in rats when carotene was fed but not when Vit. A was fed. Murray *et al.*(7,8,9) reported that addition of chlorotetracycline to Vit. A-deficient diets decreased the rate of depleting Vit. A reserve in rats, prolonged the survival period and increased the growth rate when restricted intakes of the vitamin were fed. The antibiotic did not affect significantly liver storage of the vitamin over a 4-week period or growth rate when large doses of the vitamin were offered. The increased growth rate was observed when the vitamin was administered either orally or subcutaneously.

On the other hand, Swick *et al.*(10) reported that penicillin feeding did not increase growth rate of rats receiving limited intakes of Vit. A, whereas Hartsook *et al.*(11) reported that dietary supplementation with tetracycline not only failed to exert a sparing effect on Vit. A but actually increased the deficiency syndrome.

Hence, the preponderance of evidence seem to indicate that some of the antibiotics do influence carotene and Vit. A metabolism when taken into the animal body under certain conditions. It appears that further knowledge of the physiological effect of the antibiotics in this particular connection will be helpful in explaining their mode of action in nutrient economy.

Methods. In the initial experiment weanling rats were depleted of their body-stores of Vit. A by maintaining them on the U.S.P. Vit. A-deficient diet.† All rats were caged in individual all-metal cages having raised screen floors. When depleted the rats were arranged into comparable experimental groups and fed the basal diet after incorporating into it varying amounts of chlorotetracycline (Aureomycin).‡ In addition, each rat received by mouth 1 μg beta carotene (in cottonseed oil) daily as the Vit. A supplement. Distilled water was available at all times. The rats were continued under observation for 28 additional days unless death intervened. Body weight and food intake records were recorded and external appearance and actions of the test animals noted.

When it became evident that cholorotetracycline incorporated into a Vit. A-deficient diet exerted a definite growth-promoting effect on Vit. A-deficient rats receiving a sub optimum intake of beta carotene a second series of studies was carried out in which

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* Authorized for publication on Sept. 6, 1960, as paper No. 2488 in the Journal Series of Penn. Agri. Exp. Station.

† *U. S. Pharmacopeia* XIV, 788.

‡ Furnished through the courtesy of Dr. E. L. R. Stokstad, Lederle Labs., Pearl River, N. Y.

TABLE I. Effect of Different Levels of Chlorotetracycline on Growth Rate of Vitamin A-Deficient Rats While Receiving 1.0 μg of Beta Carotene Daily.

Animal group No.	Added chlorotetracycline, g/kg diet	Rats/group ♂	Rats/group ♀	Mean wt of diet consumed,* g	Mean wt gain,* g	Diet consumed/g of gain, g
1	None	31		266	42.6 ± 3.13†	6.24
2	.250	16		276	53.0 ± 3.61	5.21
3	.500	13		272	58.1 ± 2.14	4.68
4	1.000	22		282	70.8 ± 3.80	3.98
5	2.000	11		288	65.5 ± 4.62	4.40
6	4.000	23		279	55.8 ± 3.94	5.00
7	None		27	252	36.3 ± 3.85	6.94
8	.250		18	273	40.6 ± 2.31	6.72
9	.500		12	271	42.2 ± 2.10	6.42
10	1.000		20	275	56.2 ± 2.64	4.89
11	2.000		12	271	51.0 ± 2.92	5.31
12	4.000		24	273	43.7 ± 3.16	6.24

* During 28-day test period.

† Stand. error of mean.

weanling rats were depleted of their Vit. A reserve and the depleted animals arranged into comparable groups as in the previous experiment. The rats comprising one-half of the groups were continued on the Vit. A-deficient basal diet whereas the rats of the remaining groups received the basal diet after incorporating cholorotetracycline at the rate of 1 g/kg diet. In addition, the rats comprising the respective experimental groups received measured amounts of beta carotene or Vit. A acetate ranging from 0.0 to 8.3 I.U. daily. These rats were also continued under observation for 28 additional days.

Inasmuch as the rats that received the chlorotetracycline-containing diet increased in body weight faster than did control animals and invariably consumed more food, a third experiment was carried out. Weanling male rats were depleted of their Vit. A reserve as before and arranged into 2 comparable groups. One group received the basal diet and the other received the chlorotetracycline-containing diet. Dietary allowances were restricted so as to equalize the amount of food consumed by each rat during the respective weeks of the 4-week experimental period. All rats received 1.0 μg beta carotene daily during the 28-day test period.

Results. From the results of these studies (Table I) it was evident that growth rates of the depleted rats were most favorably affected by chlorotetracycline concentrations in the diet ranging from 250 to 1000 mg/kg. Optimum concentration of the antibiotic for growth appeared to be approximately 1 g/kg of diet. While this concentration of chlorotetracycline is considerably higher than that usually employed in experimental diets it is to be noted that both male and female rats attained maximum growth at this level. Lower concentrations of the antibiotic were less effective and higher concentrations appeared to exert a depressing effect on growth. On the particular rate of carotene intake (1 μg daily) average increase in rate of growth of male and female rats over that of their respective controls was 66 and 55% respectively.

Since Murray *et al.*(8) reported that the growth rate of rats that received adequate intakes of Vit. A was unaffected by incorporating chlorotetracycline in the experimental diet a series of growth studies was carried out to establish the level of Vit. A or beta carotene intake eliciting the maximum effect of the antibiotic on growth. It may be noted (Tables II, III) that the antibiotic exerted its maximum effect where the test animal received the minimum amount of the vitamin required to maintain body weight. When 0.43 μg of beta carotene was given daily the rats that received the antibiotic-containing diet increased in body weight approximately 3 times as much as control rats. However, when 5.0 μg of the provitamin was given daily there was no marked difference in growth rates of the 2 groups of rats. Growth rates of both male and female rats from 3 different strains were similarly affected by the anti-

TABLE II. Effect of Ingesting a Chlorotetracycline-Containing Diet on Growth Response of Vitamin A-Deficient Rats when Given Varying Amounts of Beta Carotene.

Animal group No.	Added chlorotetracycline, g/kg diet	Carotene supplement, μg/daily	Rats/group ♂	Rats/group ♀	Mean wt of diet consumed,* g	Mean wt gain,* g	Diet consumed/g of gain, g
13	None	.43	8	7	207	8.5 ± 2.14†	24.35
14	1	.43	8	8	252	25.1 ± 2.42	10.04
15	None	.57	14		226	20.2 ± 2.54	11.19
16	1	.57	16		257	36.4 ± 2.67	7.06
17	None	.57		16	215	14.5 ± 1.93	14.83
18	1	.57		16	252	27.4 ± 2.61	9.20
19	None	1.00	20		265	46.6 ± 2.01	5.70
20	1	1.00	20		286	69.8 ± 2.22	4.15
21	None	1.00		14	253	36.3 ± 3.03	6.97
22	1	1.00		14	276	58.5 ± 2.93	4.72
23	None	2.00	6	6	306	56.0 ± 2.92	5.46
24	1	2.00	6	6	327	67.1 ± 2.44	4.97
25	None	4.00	11		321	87.8 ± 3.12	3.66
26	1	4.00	11		343	92.6 ± 2.86	3.70
27	None	4.00		12	322	55.7 ± 3.14	5.78
28	1	4.00		12	320	59.4 ± 2.71	5.39
29	None	5.00	6	4	328	75.2 ± 3.17	4.36
30	1	5.00	6	4	334	76.0 ± 2.96	4.39

* During 28-day test period. † Stand. error of mean.

TABLE III. Effect of Ingesting a Chlorotetracycline-Containing Diet on Growth-Response of Vitamin A-Deficient Rats While Receiving Varying Amounts of Vitamin A Acetate.

Animal group No.	Added chlorotetracycline, g/kg diet	Vit. A acetate supplement, μg/daily	Rats/group ♂	Rats/group ♀	Mean wt of diet consumed,* g	Mean wt gain,* g	Diet consumed/g of gain, g
31	None	.258	15	16	221	15.8 ± 2.39†	14.0
32	1	.258	18	13	247	31.1 ± 2.43	7.9
33	None	.516	22	14	245	31.4 ± 2.91	7.8
34	1	.516	23	13	264	47.5 ± 3.15	5.6
35	None	1.032	6	6	298	64.3 ± 4.11	4.6
36	1	1.032	7	5	327	78.1 ± 3.10	4.2

* During 28-day test period. † Stand. error of mean.

biotic. Comparable intakes of Vit. A acetate and beta carotene were equally effective in promoting growth in presence of the antibiotic.

Invariably, the amount of food ingested per gram of growth was less for the rats that received the chlorotetracycline containing diets. When dietary allowance was restricted but adequate, (Table IV) the rats that received the antibiotic-containing diet increased in body weight approximately 20% more than controls in the same period of time and on the same amount of diet.

Discussion. Diets that contained 50 or 100 mg of chlorotetracycline/kg did not prove uniformly effective in stimulating additional growth in Vit. A-deficient rats that received 1 μg of beta carotene daily. Some of the test animals of the respective groups grew faster than corresponding control animals while others showed no marked difference in growth rate. However, when concentration of the antibiotic was increased to 250 mg or more per kg of diet increases in growth response became more uniform with the result that consistent increases were observed until the antibiotic concentration reached 1 g/kg. A somewhat similar situation was observed where extremely low levels of beta carotene or Vit. A were fed. On the low levels of Vit. A intake some of the test animals died or failed to make gains in body weight during the 28-day period even though the diet contained 1 g of chlorotetracycline/kg. This

TABLE IV. Effect of Ingesting a Chlorotetracycline-Containing Diet on Growth of Vitamin A-Deficient Male Rats Receiving Controlled Food Intakes and 1 μg Beta Carotene Daily over a 28-Day Test Period.

Animal group No.	Added chlorotetracycline, g/kg diet	Rats/group ♂	Mean wt of diet consumed,* g	Mean wt gain,* g	Diet consumed/g of gain, g
37	None	10	250	40.7 ± 2.2†	6.14
38	1.00	10	250	49.9 ± 2.4	5.01

* During 28-day test period. † Stand. error of mean.

made it difficult to evaluate the effect of the antibiotic on growth or on food consumption. However, when Vit. A intake approached 0.7 I.U. daily, growth response became more uniform within each experimental group. Vitamin A intakes inadequate for sustaining life in the control animals over the 4-week test period were effective in sustaining life and often resulted in appreciable growth in rats that received the chlorotetracycline-containing diet. Furthermore, when the antibiotic was incorporated in the basal ration during the depletion period the test animals grew faster than control animals and were somewhat larger when depleted but required no additional time for depletion. Rats transferred to the antibiotic-containing diet after being depleted on the basal diet but given no Vit. A supplement died just as early as control animals subsisting on the basal diet through the entire period. Rats depleted on the antibiotic-containing diet and then transferred to the basal diet performed similarly to animals retained on the basal diet. In general, food consumption paralleled growth rate but in all instances where comparisons were possible it required less of the antibiotic-containing diet to produce a unit of body-weight gain than it did of the basal diet. While this observation has been reported many times a clear-cut explanation of the phenomenon is lacking. No consistent evidence of increased tissue storage of Vit. A was found following chlorotetracycline feeding. However, this could hardly be expected where daily intake of the vitamin was of the range employed in the present studies.

Summary. Addition of chlorotetracycline to a Vit. A-deficient diet was found to stimulate additional growth in Vit. A-deficient rats that received restricted intakes of beta carotene or Vit. A. Extent of additional growth depended on amount of the antibiotic incorporated in the basal diet and amount of the supplementary vitamin fed. Under the conditions of the experiments reported a diet that contained 1 g/kg of chlorotetracycline exerted the greatest growth-stimulating effect in rats that subsisted on a minimum maintenance intake of Vit. A or beta carotene. This increase in growth ranged from 200% in the low Vit. A-intake animals to essentially no increase in growth rate of animals that received the higher doses of the vitamin. Food requirement per unit of weight increase was lower in the rats that received the chlorotetracycline-containing diets. Rats restricted to equal food intakes made greater gains in weight when the diet contained the antibiotic.

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Received Sept. 15, 1960. P.S.E.B.M., 1960, v105.

Inhibition of Leukocyte Phagocytic Activity by Hypergammaglobulinemic Multiple Myeloma Plasma.* (26124)

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This report presents preliminary studies on a phenomenon in which the phagocytic activity of peripheral blood leukocytes in an *in vitro* system is virtually completely inhibited by hypergammaglobulinemic multiple myeloma plasma.

Materials and methods. The phagocytic activity of leukocytes is evaluated by means of a procedure used in this laboratory since 1952(1). Briefly, heparinized blood is incubated with saccharated iron† in a rotating plastic vial immersed in a water bath maintained at 37.5°C. After incubation, coverglass blood films are pulled. Saccharated iron in the leukocytes is demonstrated by means of the Prussian blue reaction and the preparations are counterstained with basic fuchsin. For each sample, a minimum of 100 potentially phagocytic cells, *i.e.*, granulocytes and monocytes, are classified individually as being "marked" (containing one or more particles of iron) or "unmarked" (containing no iron).

The ratio of volume of blood to volume of iron must be constant among the incubation mixtures(1). In the present studies this ratio is 100 volumes of blood plus one volume of iron, total volume of each sample being 2-5 ml.

If samples are aspirated from such an incubating system serially over a period of about one hour, the number of potentially phagocytic leukocytes which have failed to ingest iron is found to decrease exponentially with time. Thus, number of "marked" or "unmarked" phagocytic leukocytes per 100 potentially phagocytic cells examined may be used as an index of rate of phagocytosis.

This series of studies represents 13 patients with multiple myeloma (MM), 6 of them with hyperproteinemia due to abnormally high levels of gamma globulin. Studies on 4 patients with hypergammaglobulinemia due to disease other than M.M. are also included. Plasma protein studies were carried out in the Special Determinations Laboratory, Univ. of Rochester Med. Center. Total protein determinations were made by means of the biuret procedure and serum protein fractionation by paper strip electrophoresis.

The basic protocol utilized 4 cell-plasma-iron incubation mixtures. The patient's cells were incubated in (1) patient's plasma and (2) normal compatible plasma; cells from normal compatible blood were incubated in (3) their native plasma and (4) patient's plasma. In some instances a fifth sample was included, consisting of the patient's cells and plasma plus commercially available human gamma globulin containing 16.5 g % γ globulin (Immune Serum Globulin).‡ Cells incubated in plasma other than their native plasma were first washed once with a small volume of the plasma in which they were to be incubated.

The effect of diluting patients' plasma with

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* Dr. Scott N. Swisher and associates, Dept. of Medicine, Univ. of Rochester School of Med. and Dentistry, and Dr. Richard F. Platzer, Clifton Springs Sanatorium, Clifton Springs, N. Y. were responsible for diagnosis and care of the patients represented in this report, and called them to the author's attention. One ambulatory patient was under the care of Dr. C. B. F. Gibbs who kindly enlisted the patient's cooperation in the study. The author is grateful to these colleagues for their valuable assistance. Dr. John H. Vaughn and Dr. Vincent Butler have given invaluable consultation on this research. This report is based on work performed under contract with U. S. Atomic Energy Comm. at Univ. of Rochester School of Med. and Dentistry Atomic Energy Project, Rochester, N. Y.

† The commercial preparation, *Proferrin*, kindly made available by Sharpe and Dohm, was used in all the studies.

‡ This material was kindly made available to Medical Division, Univ. of Rochester Atomic Energy Project, by Lederle Labs.

TABLE I. *In Vitro* Phagocytosis by Granulocytes.

Patient	Serum proteins (g %) Total protein	Total globulin	Total γ glob.	% granulocytes ingesting Fe Pt. cells + Pt. pl.*	Pt. cells + D. pl.	D. cells + Pt. pl.	D. cells + D. pl.
			M.M. with hypergammaglobulinemia				
O.R.	10.9	—	6.0	0	—	—	—
P.P.	13	11.5	10.7	0	99	0	51
M.L.	9.7	7.8	6.8	1	100	1	99
N.F.	10.6	8.5	6.4	2.5	90	3.5	98.5
F.S.	12.0	9.3	7.5	0	100	1	87
B.A.	11.2	9.3	8.0	1	83	0	80
			M.M. with increased α or β globulins				
N.W.	9.4	6.1	.4	84	98	90	88
P.C.	10.9	8.7	<.1	90	100	—	—
M.S.	9.7†	6.7	.3	80	94	46	79
S.W.	8.1†	5.2	1.0	100	100	100	99
			M.M. without hyperproteinemia				
I.O.	7.0	2.1	.4	93	93	89	87
G.B.	6.3	2.6	.7	97	99	97	96
E.W.	6.8	2.9	.9	64	76	98	96
			Absolutely or relatively increased γ globulin; not M.M.				
M.M.[1]	7.7	4.2	2.4	100	95	91	98
J.R.[2]	6.1	4.3	1.5	91	98	93	75
G.M.[3]	5.6	3.3	1.6	65	100	86	100
K.B.[4]	9.4	7.0	5.7	78	87	94	98

* Pt. = patient's; pl. = plasma; D. = normal compatible donor's.

† Protein determinations on plasma instead of serum.

[1] Sjogren's syndrome; [2] chronic alcoholism and CNS atrophy; [3] lymphoma; [4] chronic liver disease.

(1) 0.85% NaCl solution and (2) normal compatible plasma was studied in experiments representing 3 myeloma patients with hypergammaglobulinemia.

The possibility that multiple myeloma plasma might simply interfere with the Prussian blue reaction was evaluated as follows: Cells which had been incubated with iron in M.M. plasma were washed twice with normal plasma and suspended in normal plasma before smears were pulled. No intracellular iron was demonstrable in these cells. The counterpart of this experiment was also carried out, *i.e.,* cells which had been incubated with iron in normal plasma were washed twice with multiple myeloma plasma and suspended in it before smears were pulled. The intracellular iron in these cells was demonstrated just as vividly as in cells which remained in normal plasma.

Results. Data are summarized in Tables I, II, III and IV. The salient points are as follows: (1) Hypergammaglobulinemic multiple myeloma plasma inhibits, almost completely, *in vitro* ingestion of saccharated iron by peripheral blood phagocytes from both healthy individuals and M.M. patients (Table I). (2) Plasma of M.M. patients with hyperproteinemia due to increased amounts of α or β globulins rather than γ globulins does not inhibit ingestion of saccharated iron by peripheral blood phagocytes, nor is phagocytosis inhibited by plasma of multiple mye-

TABLE II. Effect of Diluting M.M. Plasma with Saline or Normal Plasma. Patient, F.S. Total protein, 12 g %. γ globulin, 7.5 g %.

	% patient's plasma	% diluent	% marked phagocytes*
Saline diluent	100	0	8
	80	20	13
	40	60	83
	20	80	78
	10	90	52
	5	95	33
Normal plasma diluent	100	0	3
	80	20	5
	40	60	68
	20	80	80
	10	90	81
	5	95	86
	0	100	91

* Cells containing 1 particle or more of saccharated iron.

TABLE III. Effect of Adding Immune Serum Globulin (ISG) to Patients' Cells and Plasma and Saccharated Iron *In Vitro*.

	Serum proteins (g %)			% marked phagocytes in:		
Patient	Total	Globulins	γ glob. only	Plasma only	Plasma + ISG	g % γ glob. after adding ISG (calculated)
		M.M.* with increased γ globulin				
F.S.	12	9.3	7.5	0	1	9.4
N.F.	10.6	8.5	6.4	2.5	6.5	8.7
B.A.	11.2	9.3	7	1	2	8.9
		M.M. with increased α or β globulin				
N.W.	9.4	6.1	0.4	85	87	2.6
M.S.	9.7	6.7	0.3	80	9	2.4
		M.M. without hyperproteinemia				
I.O.	6.3	2.6	0.7	89	3.5	2.1
G.B.	6.8	2.9	0.9	99	26	2.2
E.W.	7.0	2.1	0.4	93	7	3.6
		Absolutely or relatively increased γ globulin; disease not M.M.				
M.M.	7.5	4.0	2.4	95	88	5.4
J.R.	6.1	4.3	1.5	91	20	4.2
G.M.	5.6	3.3	1.6	65	23	4.4

* Multiple myeloma.

loma patients with normal total plasma protein levels (Table I). (3) Hypergammaglobulinemic M.M. plasma diluted with normal plasma or saline is entirely capable of supporting brisk phagocytosis. Results representing the two types of dilution studies may be compared in Table II. The percentage of phagocytes ingesting saccharated iron increases progressively as the proportion of normal plasma increases, reaching a maximum in 100% normal plasma. Diluting the abnormal plasma with saline also increases phagocytosis. In saline dilutions, however, a minimum of about 20% patient's plasma appears to be necessary for optimum phagocytic activity. Although results of plasma dilution studies for 3 hypergammaglobulinemic M.M. plasmas studied in this way are similar, further studies must be completed to determine how consistent the shapes of the dilution curves may be from one plasma specimen to another. (4) Addition of commercially available immune serum globulin to the plasma of multiple myeloma patients with hypergammaglobulinemia fails to promote ingestion of saccharated iron by granulocytes (Table III). Furthermore, phagocytosis is depressed by addition of normal gamma globulin to multiple myeloma plasma which is not hyperproteinemic or in which hyperproteinemia is due to increased amounts of alpha or beta globulins rather than gamma globulins. Addition of normal γ globulin to normal plasma inhibits ingestion of saccharated iron by phagocytic leukocytes only in concentrations greater than about 5 g% (Table IV). This is in distinct contrast to the concentrations of only 2 to 3 g% required to inhibit phagocytosis in M.M. patients without hyperproteinemia. (5) Incubating particles with commercial immune serum globulin before exposing them to blood cells *in vitro* did

TABLE IV. Effect of Adding Immune Serum Globulin (ISG) to Normal Blood and Saccharated Iron *In Vitro*.

	% marked phagocytes		
Normal donor No.	Plasma only	Plasma + ISG	γ glob. after adding ISG*
1	88	100	3
2	89	96	3
3	100	99	3
4	95	88	1.2
	95	85	1.6
	95	32	4.5
5	99	60	3
	99	64	3.4
	99	43	4
	99	10	5

* Protein fractionation studies were not carried out on earlier normal plasma samples. These are assumed to contain approximately 1 g % γ globulin. Normal range in Special Determinations Laboratory is 0.6-1.7 g %.

not inhibit phagocytosis in the few instances in which it was tested.

Discussion and conclusions. This report is intended to describe a new observation. Speculation about the ultimate significance of virtually complete inhibition of leukocytic phagocytosis in hypergammaglobulinemic multiple myeloma (M.M.) plasma *in vitro* is clearly unwarranted at present, but a few tentative conclusions are justified: (1) Depressed phagocytic activity is directly attributable to the γ globulin fraction in M.M. plasma and not to a cellular defect. (2) Plasma from M.M. patients with hyperproteinemia due to increased levels of α or β globulins, or from patients with hypergammaglobulinemia due to disease other than M.M. does not inhibit leukocyte phagocytic activity. (3) Inhibited phagocytosis appears not to be solely a function of total gamma globulin level. This is most clearly demonstrated by the relatively low levels of γ globulin which inhibit phagocytosis in plasma of M.M. patients without hypergammaglobulinemia or hyperproteinemia; most notably when γ globulin levels are lower than normal. (4) Since the inhibitory effect of hypergammaglobulinemic M.M. plasma may be decreased or abolished by diluting the abnormal plasma with normal plasma or isotonic saline, the effect is not due to a simple deficiency. Specifically, the inhibitory phenomenon appears not to be due to a deficiency of normal gamma globulin. (5) Studies of the phenomenon are continuing.

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Received Sept. 19, 1960. P.S.E.B.M., 1960, v105.

Some Characteristics of Para-Mucus Secretion.* (26125)

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It has been reported from this laboratory that the topical application of iodoacetamide (IAA) or N-ethyl maleimide (NEM) solutions to the mucosa of canine gastric pouches will evoke a flow of mucinous secretion for many hours(1). This material had never been recognized before, and because its physiological significance is still unknown, it was designated "para-mucus"(2). Exploratory experiments on the pepsin content of para-mucus indicated that the zymogen is present in appreciable quantities, and that it is probably a significant component in chemical characterization of this mucinous secretion. This report presents a systematic study of the variations in pepsinogen content of para-mucus, using specimens collected fractionally every 30 minutes for 6 hours following topical application of IAA solution to the gastric mucosa in dogs with a Heidenhain pouch and antrectomy. Several other characteristics of this material are presented here.

Material and method. Four Heidenhain pouch dogs, provided with an antrectomy, were used in these experiments. The antral resection was performed to insure the virtually complete absence of HCl secretion in the fasting state, in accordance with previous experience in this laboratory(3). Each dog was subjected to the following 2 types of experiment: A) *Histamine stimulation*: Dogs were starved for at least 18 hours before experiment, and unstimulated (basal) secretion was collected for 2 hours, at 60-minute intervals. Following this, histamine was administered subcutaneously (0.1 mg of histamine

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* This work was supported by grant from Nat. Cancer Inst., U.S.P.H.S.

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TABLE I. Volume-Rate of Flow and pH of Basal and Histamine-Stimulated Gastric Secretions.

	Basal secretion		Histamine-stimulated secretion			
			1st hr		2nd hr	
Dog No.	Vol-rate* (ml/hr)	pH	Vol-rate (ml/hr)	pH	Vol-rate (ml/hr)	pH
326	1.3	7.2	6.8	1.0	1.8	1.5
334	1.9	7.2	2.1	6.6	1.5	7.0
	1.0	7.4	2.6	3.5	2.0	6.8
	1.0	7.2	4.0	3.5	2.5	6.8
	1.0	7.0	4.5	3.5	1.3	6.5
340	.8	7.2	7.2	1.0	3.6	1.0
	.8	7.0	13.5	1.0	8.5	1.0
	1.3	7.0	10.5	1.0	3.2	1.0
382	1.4	7.5	8.3	1.0	5.5	1.3
	1.3	7.0	3.8	1.2	4.4	1.4
	1.8	7.2	10.0	1.0	3.0	3.5
Mean	1.2 (.8-1.9)†		6.66 (2.1-13.5)		3.39 (1.3-8.5)	

* Mean volume-rate over a 2-hr interval.
† Range.

base/kg body weight) and the acid secretion was collected for two 60-minute intervals thereafter. Such experiments were done to be sure that the acid-secretory behavior of the dogs was normal. B) *Topical application of IAA*: IAA in water solution, at a concentration of 3mM (55.5 mg/100 ml) was applied topically for 30 minutes, and secretion was collected every 30 minutes for 6 hours (12 experiments). The technics were those described previously(1).

For each specimen, volume, pH (electrometric), and pepsinogen (hemoglobin substrate method of Anson and Mirsky, as modified by Bucher) were determined as usual. Activity of the enzyme was expressed as meq. tyrosine × 10^4-designated Hb-units. Total and organic solids were also determined in 4 of these experiments, using the method previously described(4).

Observations and comment. Basal secretory activity. Volume-rate of secretion during basal conditions ranged between 0.8 and 1.9 ml/hour, with an average of 1.2 ml/hour for 2 hours of collection (Table I). There were no striking differences among the several dogs as to volume secreted, size of pouch, or lapse of time after operation. pH of these basal specimens never fell below 7.0.

Response to histamine stimulation. In 3 of the dogs, histamine produced the usual pattern of acid secretion during the first 2 hours after injection (Table I). In the fourth animal (dog #334), the volume secreted during 2 hours after histamine was markedly lower than for the other 3 averaging only 2.1 times as great as during basal secretion, in contrast to a 5.2-fold difference for the others. The pH of this dog remained above 3.5 in 6 of the 8 hourly collections, suggesting some abnormality of acid secretory function in this animal. Even doubling the histamine dosage failed to evoke any marked output of free acidity.

Volume and pH response after IAA. During the first 3 half-hours after topical application of IAA, volume-rates of para-mucus secretion were extremely high as compared with such data for basal and even acid secretion after histamine stimulation. For this interval, the rates varied in the range 4.6-11.5 ml/half hour with a mean of 7.2 for all 12 experiments. Since the responses to IAA of dog #334 were consistent with those for the other 3 animals, they were included in this average. By plotting the average volume-rate of para-mucus secretion, in ml/half hour, as a function of time following IAA stimulation and drainage (Fig. 1), it appears that the data conform roughly to a straight line with negative slope. For the sixth hour, mean volume-rate is still nearly 4 times that observed during the basal period, suggesting a persistence of IAA activity for at least this length of

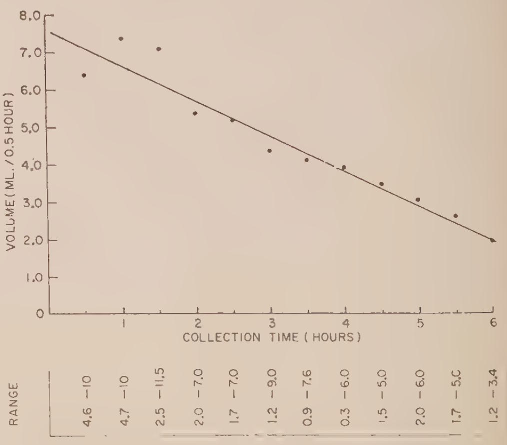

FIG. 1. Volume-rate of secretion of para-mucus as a function of time following topical application of iodoacetamide solution for 30 min. (mean of 12 exp. with 4 Heidenhain pouch dogs).

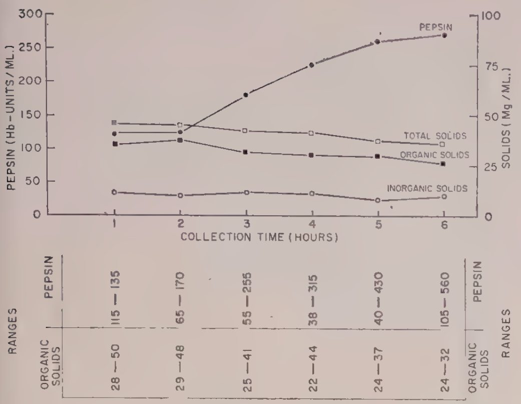

FIG. 2. Concentrations of several constituents of para-mucus as functions of time following topical application of iodoacetamide solution for 30 min. (mean of 12 exp. with 4 Heidenhain pouch dogs).

time, and probably one or more hours longer.

As previously reported(1), the pH values varied in the range 7.0-7.4 for all 30-minute specimens of the 12 experiments. These minor variations were not correlated with volume-rate of secretion nor with time of collection.

Because of the possibility that the secretory response to topically applied mustard oil (allyl iso-thiocyanate) bears some relation to that following stimulation with IAA, volume-rate curve for this mucinous secretion (5) was compared with that in Fig. 1. Whereas the latter approximates a rectilinear relation, the former was semi-logarithmic. The highest initial volume-rate of secretion following mustard oil administration was 17.4 ml/15 minutes; the mean for 15 experiments was 11.1 ml/15 minutes. These rates are equivalent to 34.8 and 22.2 ml/half hour respectively, the latter value being over 3 times as great as the mean rate (7.2) observed during the first 3 half-hour collection periods in the present experiments.

Organic and inorganic solids in para-mucus. In 4 of the experiments in which a single topical application of IAA was employed, the contents of total organic and inorganic solids were determined in each hourly sample. The resulting data, expressed as concentrations in terms of mg/ml secretion, are summarized in Fig. 2 as a function of collection time. The concentration of inorganic substances remained practically constant around a mean level of 9.6 mg/ml (unweighted for volume) throughout the 6 hours of experiment. Range for all 24 specimens was 5-14 mg/ml. Concentrations of organic and total solids, however, showed a consistent downward trend, starting with the second hour of collection; this pattern was followed by the 4 individual experiments as well as the curve for their average. For total solids the range of these 6 mean values reflects a mean change of 22%; for organic solids alone it is 26%. The individual values comprising these means varied from 22 to 50 mg/ml for organic solids, and from 34 to 57 mg/ml for total solids. Hourly outputs of all 3 of these factors, plotted as mg/hour against collection time (Fig. 3), reveal an even sharper descending line than do the corresponding concentration data of Fig. 2. Mathematically, this finding implies a definitive correlation with collection time, but also with volume-rate of secretion as is evident by comparison with the histogram for the latter. Whether the correlations for output of solids/hour with volume-rate/hour have physiological significance beyond that indicated by the corresponding concentration graphs, or whether they are entirely spurious, cannot now be established.

Concentration and rate of secretion of pepsinogen. A) Basal secretion: Pepsinogen concentration in the fasting secretion from 10 experiments ranged between 15 and 120

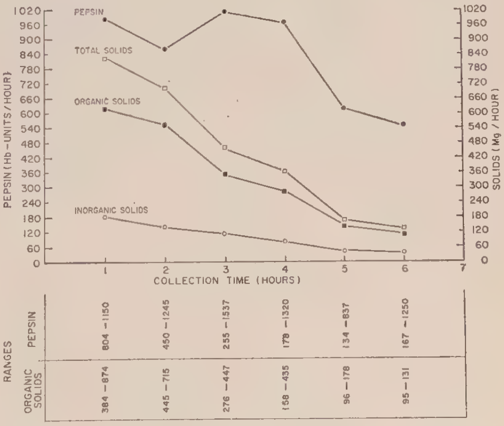

FIG. 3. Output-rates of secretion of several constituents of para-mucus as functions of time following topical application of iodoacetamide solution for 30 min. (mean of 12 exp. with 4 Heidenhain pouch dogs).

(mean 62) Hb-units/ml. Because of the low volumes of juice, however, rates of zymogen secretion (outputs) were also very low, varying in the range 8-94 (mean 40) Hb-units/half-hour. These data reflect the average behavior over 2 hours of observation.

B) After histamine stimulation: In the 3 dogs which gave acid secretion (pH in the neighborhood of 1), both concentrations and outputs of pepsinogen showed striking increases after injection of this stimulus. Thus, for individual hourly samples of all 10 experiments, concentrations were 3 or 5 times as great as those in the related basal secretion, though occasional specimens of histamine secretion had less than control values. The highest such concentration was 225 Hb-units/ml; the lowest was 25. Because of the great differences in volume-rate of flow after histamine, the data for output/hour showed even more striking rises over pre-injection levels. Thus, 10 to 35-fold increases were obtained in a number of the specimens, and in no case was post-injection level less than that for the basal secretion. The over-all range of post-histamine output values in the 3 good secretors was 184-2000 Hb-units/hour. In the fourth dog—the poor secretor for which the pH never fell below 3.5—individual pepsin concentrations and outputs following histamine were generally lower than in the other 3 animals, but the various ratios were in no way different.

C) After IAA application: Concentration of pepsin (Fig. 2) rose progressively for the 6 hours following drainage of the IAA solution—except from the first to the second hour—while volume-rate of secretion decreased steadily. During the fifth and sixth hours, when the pepsin curve started to flatten out, the enzyme concentrations averaged more than double those in the first 2 hours. The range of individual values in the 24 specimens making up this graph was 38-560 Hb-units/ml. Output of enzyme per hour (Fig. 3) decreased but slightly (if at all) during the first 4 hours, but thereafter began to drop significantly. The over-all range of variation among individual hourly specimens was 134-1755 Hb-units/hour. The curve for this variable appears not to be closely related to the corresponding one for output of total solids in the same figure, which suggests that pepsinogen probably comprises only a minor fraction of total content of organic substances in the IAA para-mucus. However, output ratios for pepsinogen to organic solids (corresponding to quantity of potential peptic activity per mg organic substances) increases steadily with time. The time curve for this ratio (Fig. 4), derived as averages from the individual ratios, conforms generally to the pattern of time-curves for enzyme concentration per ml of secretion in Fig. 2. This similarity results from the nearly constant character of the concentration of organic solids in Fig. 2. For the individual samples, ratios varied in the range 0.40 to 11.36 Hb-units/mg organic substances. Even in the low-acid secreting dog (#334), pepsinogen output and concentration after IAA stimulation were similar to those in the other 3 dogs. The individual graphs representing pepsinogen output as a function of time were similar for these 3 animals; only the low secretor showed an elevation at the second, third and fourth hours, apparently as a delayed response to the stimulus.

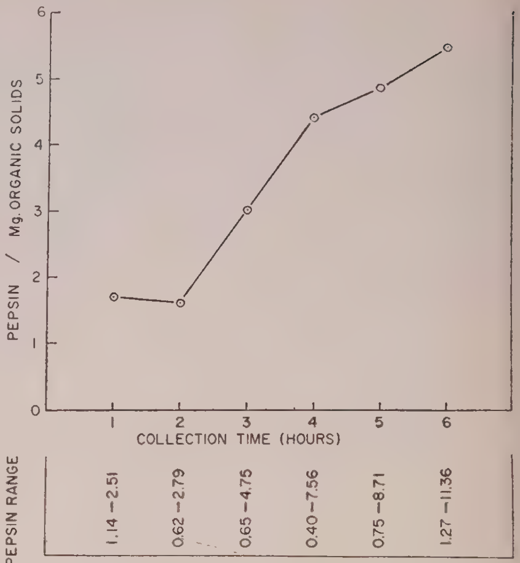

FIG. 4. Pepsin activity/mg organic solids as a function of time following topical application of iodoacetamide solution for 30 min. (mean of 4 exp. with 4 Heidenhain pouch dogs).

Comparison of all these data for the 4

dogs with corresponding values for basal and histamine-stimulated specimens revealed that outputs of pepsin for the mucinous secretion collected during the first 2 hours after IAA application were considerably higher (at least 2-fold) than for these other types of secretion. This observation may be significant in regard to the problem of whether histamine acts as a stimulus to pepsin as well as to acid secretion, or whether the enzyme is merely "washed out" by the parietal fluid. A study of enzyme response to histamine injected simultaneously with topical application of IAA may supply important evidence toward solving this problem.

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Received Sept. 12, 1960. P.S.E.B.M., 1960, v105.

Ultrastructural Abnormalities In Whipple's Disease.* (26126)

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Whipple's Disease has been recognized with increasing frequency as one of the clinical entities associated with intestinal malabsorption. The underlying etiology has remained obscure but the histopathology of this disorder has been defined more accurately as a result of a variety of technical advances. Whipple(1) noted a prominence of fatty deposits in extracellular spaces as well as within tissue mononuclear cells. He also found many macrophages with foamy cytoplasm which did not stain for fat. Black-Schaffer subsequently demonstrated that these latter cells stain strongly with the periodic acid-Schiff (PAS) stain(2). His observations have been amply confirmed, and the occurrence of these PAS positive cells in the connective tissue throughout the body has been appreciated(3,4,5).

The introduction of peroral biopsy instruments(6,7) has made possible the histologic examination of normal and diseased small intestinal mucosa unaltered by post-mortem autolysis. This technic also permits tissue to be obtained rapidly enough for fixation and preservation of the fine structure for electron microscopic studies. This is a preliminary communication of light and electron microscopic findings in an intestinal biopsy from a patient with previously proven Whipple's Disease.

Methods. A fasting jejunal biopsy was obtained by means of a Crosby capsule from a 65-year-old male with known Whipple's Disease.† Portions of the specimen were fixed within one minute after biopsy in buffered osmium tetroxide(8) and in Dalton's solution (9), then dehydrated and embedded according to standard procedures(10).

Portions of the biopsy were also fixed in formalin for light microscopy. Sections of the osmium fixed as well as formalin fixed material were stained with the periodic acid-Schiff reagent. Thin sections were examined in an RCA-EMU 2B electron microscope at original magnifications of 1200 to 10,000 ×.

Results. By light microscopy the lamina propria of the biopsy specimen was found to contain a dense population of macrophages filled with PAS positive granules (Fig. 1). The granules appeared to be smaller in the cells adjacent to the lining of the epithelial

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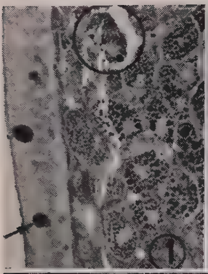
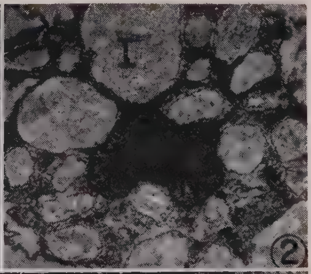
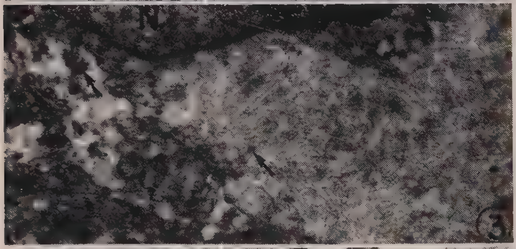
* This is publication No. 284 of Robert W. Lovett Memorial Unit for the Study of Crippling Disease. Supported by grants from Nat. Inst. of Arthritis and Metab. Dis., Nat. Inst. Health, U.S.P.H.S.

† This patient was Case 46112, Case Records of Mass. Gen. Hosp., *New England J. Med.*, 1960, v262, 572.







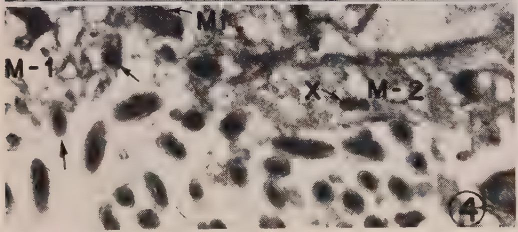


FIG. 1. Periodic acid-Schiff (PAS) stain of osmium material obtained by peroral intestinal biopsy. The lamina propria is filled with macrophages that contain numerous PAS positive granules. Mucus secreting cells in epithelium (arrows) also are strongly PAS positive. One macrophage, comparable to that in the circle, is seen in Fig. 2. ×225.

FIG. 2. Electron micrograph of a macrophage which, by light microscopy, was loaded with PAS positive granules. The large pale inclusions (I) indent nucleus (N) and fill cell cytoplasm. Within these inclusions are fine membranous structures. ×2800.

FIG. 3. Electron micrograph of a macrophage inclusion body adjacent to nucleus (N). Within the inclusion are a series of membranes, vesicles and dense granules (arrows). ×9000.

FIG. 4. Electron micrograph of cell borders of 2 adjacent macrophages (M-1 and M-2) that were located immediately subjacent to epithelium. Ovoid bodies (arrows) with outer limiting membranes, central dense areas enclosing a small pale core impinge upon cytoplasm. At the arrow at extreme left an ovoid body is seen to indent the cell membrane, while that to its right is almost completely enclosed by 2 arms of cytoplasm. A mitochondrion (M1) is seen in one macrophage and a dense body (X) without an outer limiting membrane can be observed in cytoplasm of the second macrophage. ×10,800.

cells than in those lying deeper in the lamina propria.

Successive thick and thin sections cut on the Porter-Blum microtome allowed identification in the electron microscope of the PAS positive inclusions of light microscopy. These inclusions were complex and varied in structure. In general, they consisted of series of closely packed membranes, granules, and vesicles (Fig. 2). The membrane-containing inclusions varied in size, and though they usually appeared to be discrete and separate, in several areas possible interconnecting channels were visualized. The majority of the large inclusions contained short curved membranes. The small granules were sometimes seen along the membranes and in other instances were formed in small clusters. Vesicles and tubules were also present (Fig. 3). Other less finite structures, some dense and some amorphous appeared in these large inclusion bodies.

Along the edges of the macrophages, rare microvilli were observed. Dense round, oval and rod shaped bodies were seen in close apposition to these cells, especially, but not solely near the epithelial lining. These dense bodies had a discrete structure with an outer limiting membrane, a less dense "jacket," and inner dark body with a pale core (Fig. 4). In several sections these bodies indented the cell border, were surrounded by cytoplasm, and finally appeared singly or in groups as fully "phagocytized" particles. Their average outermost diameter measured 250 mμ.

The "brush border" of the epithelial cells of the biopsy specimen had normal appearing microvilli, whose average length was 750 mμ and diameter was 100 mμ.

Discussion. In Whipple's disease the principal changes recognized in the small intestinal mucosa consist of dilation of lymphatic spaces, and accumulation of foamy macrophages in the lamina propria. Since the occasional lipid-filled macrophages are rather non-specific, it is the non-lipid, PAS positive material which is considered characteristic of the disease.

The present observation that PAS positive material represents a complex membrane system is of interest in the light of what is known of the biochemistry of the PAS reaction and previous electron microscopic observations on other PAS positive material. It was first believed that the positive PAS reaction of the macrophage indicated the certain presence of glycoprotein. However, since this reaction depends upon oxidation of 1,2 glycol groups (as in carbohydrates) to form aldehydes, not only glycoprotein, but other substances containing unsaturated lipids, phospholipids, etc. would be expected to react. Thus the PAS reaction *per se* does not provide positive identification of the reacting material(11).

A number of electron microscopic observations have also demonstrated the diversity of PAS positive material. For example, PAS positive mucin in the epithelial cells of the intestinal border have a grey, homogeneous and structureless appearance, while large round PAS positive bodies of fetal kidney contain both amorphous material and concentric lamellar structures(12). In the present study, the large PAS positive inclusion body seemed to contain a variety of forms, *i.e.,* large and small granules, vesicles and membranes, the last predominating.

Although macrophage fine structure has occasionally been commented on, few detailed observations are available. It has, however, been observed that phagocytized material of many different types contains laminated structures ("myelin figures") when viewed in the electron microscope(13,14,15,16). However, it appears that the present large inclusion bodies with their sinuous membranes and granules are a unique finding in this patient with Whipple's disease. It will be important to determine whether these inclusions represent abnormal products of the metabolism of the cell itself or are the result of ingestion of an unusual substance.

The nature of the dense bodies observed at the macrophage cell border is unknown. Their increased numbers near the epithelium are suggestive of material being absorbed from the intestine and engulfed by the macrophages. Although the fasting state of the patient does not rule this out, the particles do not have the characteristics of absorbed fat as described by Palay(17,18). Although virus particles and inclusion bodies have been observed with dimensions and appearance similar to the present dense bodies(19,20), no conclusion concerning the possibility that these particles represent a virus can be drawn from the electron microscopic data presented. Further investigations are being carried out.

In several preivous studies of intestinal mucosa in patients with malabsorption due to non-tropical sprue, abnormally short microvilli have been observed by electron microscopy(*21,22*). It is interesting that in this patient with Whipple's disease and malabsorption, the microvilli in the samples studied were of normal dimensions, suggesting that malabsorption may occur in presence of normal microvilli.

Summary. A fasting jejunal biopsy specimen, obtained from a patient with Whipple's disease, was studied by light and electron microscopic technics. Periodic acid-Schiff positive inclusions in the macrophages were found to have a complex ultrastructure consisting of a series of membranes, vesicles and granules. Dense bodies of unknown nature were observed in close approximation to the macrophage cell border, and within its substance. The virus-like character of these particles has been discussed.

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Received Sept. 21, 1960. P.S.E.B.M., 1960, v105.

Influence of Total Body X-irradiation on Tumor Induction by Parotid Tumor Agent in Adult Mice. (26127)

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The parotid tumor agent, (PTA, polyoma virus), has certain limitations of action in several parameters, such as species, age, mouse strain, and tissue susceptibilities and type of response by different cells or by cells of the same morphogenetic lineage infected at different levels in their development. Differences in cell sensitivity and specificity of response to PTA have been discussed(1).

In a previous study(2), sensitivity in certain strains of mice to PTA was demonstrable through the first 14 days *post partum* without appreciable lengthening of the latent period. Beyond this age the response has not been adequately studied. Preliminary data from our laboratory indicated that sensitivity was increased in 1 to 2-month-old mice given whole-body X-radiation prior to injection of virus. The present report concerns the response of adult mice of several age groups to PTA, influence of whole-body X-irradiation on this response and a histologic comparison of the tumors and lesions in mice inoculated as adult or as newborns.

Methods and materials. Mice. C3Hf/$_{\mathrm{BiLw}}$ mice from our pedigreed colony were used. This strain was obtained directly from Dr. J. J. Bittner in 1955 and has been inbred continuously since that time, brother x sister. Frequency and types of neoplasms in a sample of mice of this subline have been reported (2). Parotid tumors within the breeding colony or following acute whole-body-X-irradiation have been observed only very rarely (3), despite a high frequency of serologic convertors(4). Age of these mice at radiation and injection of virus is given in the results.

Virus. PTA has been passaged in the continuous tissue culture line derived from a lymphocytic leukemia, designated P-388D$_1$. The origin of virus and cell strain have been described(2,5). Source of virus was the tenth serial passage obtained by collecting tissue culture fluid at time of maximum CPE. This material had been stored at −70°C for 8 months prior to use here. The HA titre of the 10-fold diluted material, used for intravenous inoculations in adult mice was 1:40. Intravenous injection of 0.5 ml of virus was accomplished in Groups 1 and 2 (Tables I and II) through the lateral tail vein. This represented an inoculum of $10^{4.9}$ adult mouse ID$_{50}$/mouse as determined by the MAP test(6). Although HA titre and mouse ID$_{50}$ are relatively low, this preparation has proved to be highly oncogenic and primarily thymotrophic, inducing thymic epithelial tumors in nearly all mice inoculated at birth (7).

The adult C3Hf/$_{\mathrm{BiLw}}$ mice in the third group, (Table IV) received either tissue culture explants of salivary gland tissue or tissue culture fluid plus cell scrapings from these cultures. Explants of salivary gland tissue were exposed to tenth passage P-388 D$_1$- passaged PTA prior to explanting as gelatin-sponge-matrix cultures as previously described(8). Transfers of explants or tissue culture fluids were made on the 48th day. (See(8) for morphologic changes observed *in vitro* in these intact fragments of salivary

TABLE I. Induction of Tumors in C3Hf/$_{\rm BiLw}$ Adult Mice with Parotid Tumor Agent, PTA.

Treatment	No. of mice		No. litters (pos./total)	No. parotid tumors	Mean latent period in mo (range)	Tumor sites other than salivary
	Initial	Alive after 1 mo				
X-radiation	23	15	0/3	None	—	—
PTA	23	23	0/3	”	—	—
X-radiation + PTA	22	16	3/3	7*(43.8%)	8.7 (4½-15½)	Thymus—1 Hair follicle—1 Subcut. connective tissue—1

* Four mice had unilateral parotid tumors.

Two mice of the first group had neoplasms of reticular tissues as follows: 1 lymphocytic leukemia (15 mo), 1 reticulum cell sarcoma (14 mo); one mouse of the second group had a lymphocytic leukemia (14 mo).

TABLE II. Induction of Tumors in Thymectomized, Adult C3Hf/$_{\rm BiLw}$ Mice Following Intravenous Injection of Parotid Tumor Agent, PTA (Polyoma Virus).

Treatment	No. of mice		No. litters (pos./total)	No. parotid tumors	Mean latent period in mo (range)	Tumor sites other than salivary
	Inoc.	Alive after 1 mo				
X-radiation + PTA	41	35	5/5	10*(28.6%)	7.9 (3-14½)	Hair follicle—3 Thyroid—1 Bone—2 Mammary—3

* Five mice had unilateral parotid tumors (right or left).

Six mice had neoplasms of reticular tissues, as follows: 1 granulocytic leukemia (14 mo), 2 lymphocytic leukemia (13, 14 mo), 3 reticulum cell sarcomas (14, 15 and 15 mo). These were not associated with parotid tumors.

gland tissue exposed to PTA). Explants were transferred under the renal capsule or into the subcutaneous connective tissues or to both areas and tissue culture fluids plus scrapings of cells were introduced into the subcutaneous connective tissues of the right axilla. Each animal received fluid and cells of one culture, both in test and control series. Comparable materials from control cultures were introduced into 10 adult mice. All mice had received 300 r whole-body X-irradiation 2 days before introduction of materials.

Radiation. Two X-ray tubes placed opposite each other were operated at 200 kvp and 15 ma.; the filtration achieved was with 0.25 mm Cu and 0.55 mm Al. Radiation distance was 54 cm from each focal spot to the center of the mouse. Dose rate was 120 r/min. Mice in Group 1, (Table I) were given 300 r whole-body X-irradiation at 2 months of age, Group 2 (Table II) at 6 weeks of age; this group was thymectomized at 1 month of age, and Group 3 mice (Table IV) were nearly equally divided into 1 month and 2 month-old mice at time of receiving 300 r, whole-body X-irradiation. Virus and materials containing virus were introduced 2 to 3 days after irradiation.

Autopsies. Necropsies as described earlier (2) were performed on most of the animals in this study. Tissues were fixed in either Zenker-formol or Tellyesniczky's fluid, and stained with hematoxylin and eosin.

Results. The results in C3Hf/$_{\rm BiLw}$ mice, 2 months old at time of treatment, are given in Table I. Whole-body X-irradiation or virus alone were ineffective in inducing parotid gland tumors. These mice have now lived for 17 months. Whole body X-irradiation (300 r), followed by intravenous injection of 0.5 ml of virus resulted in typical pleomorphic parotid gland tumors in 2 of 7 males and 5 of 9 females at a considerably lengthened latent period. Four of these appeared as unilateral parotid gland tumors only, at 4½, 7, 10 and 15½ months, respectively. The relative resistance of adult mice is reflected further in the rare occurrence of other primary sites of tumors and/of lesions.

Ten of 35 C3Hf/$_{\rm BiLw}$ mice thymectomized

TABLE III. Tumors and Lesions in Twenty Non-irradiated (C3Hf × AKR)$_{F_2}$ Mice Inoculated as Newborn with Tenth Tissue-Culture Passage of PTA.*

Site	Type of reaction	No. mice showing reaction	Comments
Parotid glands	Tumor	19	All tumors microscopic size and in most instances multifocal.
Sublingual glands	”	17	
Submandibular glands	”	6	
Lacrimal glands	”	11	Only 1 tumor grossly visible at necropsy.
Thymus	”	20	All tumors grossly evident and associated with signs of respiratory insufficiency. Tumor also present in 2 ectopic thymuses.
Thyroid gland	”	13	
Tracheal submucosal glands	”	9	
Glands of oro-pharynx and nasal mucosa	”	6	All tumors microscopic in size.
Harderian gland	”	1	
Mandible	Tumor (ameloblastoma-like tumor)	1	
Renal cortex	Alteration of tubular epithelium	Marked 15 Moderate or mild 5	

* These mice represent the first 20 consecutive animals to come to autopsy out of a total of 118 that received a subcut. inj. of 0.05 ml of 1:100 dilution of culture fluid. This virus preparation was the same used to inoculate all other animals in this report, but dosage is only 1/100th that used intrav. in adult mice. Interval between inoculation and autopsy for these 20 mice ranged from 16 to 40 days.

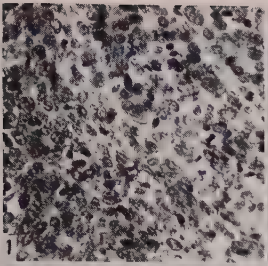
TABLE IV. Tumors in Adult C3Hf/$_{BiLw}$ Mice Receiving Organ Explants or Tissue Culture Fluids of PTA-Infected Salivary Gland Tissue.

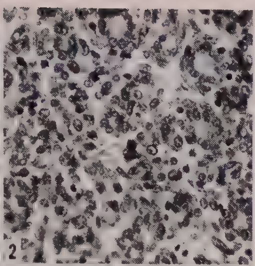
Material	No. of mice	No. of parotid tumors	Latent period in mo (range)	Tumor sites other than salivary in parotid-tumor bearing mice	Primary tumor sites in mice not bearing parotid tumors
Salivary gland explants	10	7*	4 (3-6)	Thymus, hair follicle, bone, mammary, thyroid, subcut. conn. tissue, other salivary glands	None
Tissue culture cells	20	3†	13.3 (10-18)	Subcut. conn. tissue	Thymic epithelial tumors—2 Lipo sarcoma—1 Bone—1

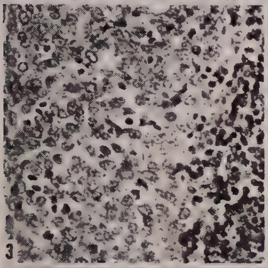
* Bilateral parotid tumors involving also other salivary glands.
† Unilateral parotid tumors only.

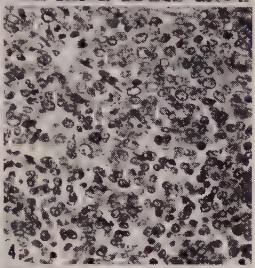
at 1 month of age and given 300 r whole-body X-irradiation and PTA at 6 weeks of age presented tumors of the parotid gland (Table II). The frequency and latent period were strikingly similar to those observed in intact adult mice, despite the fact that this preparation of virus is thymotrophic when introduced into mice less than 48 hours of age, inducing thymic epithelial tumors in nearly all recipients with involvement of the salivary gland tissues and other primary sites seen upon microscopic examination.

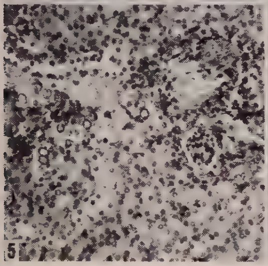
In this second group of adults, pleomorphic parotid tumors were nearly equally distributed among the sexes. Five tumors were unilateral only with no other primary sites of tumor. Tumors of primary sites in these mice with bilateral parotid tumors were indeed infrequent (Table II) in comparison with the response of neonatal mice.











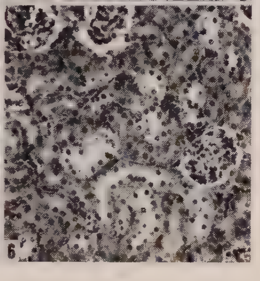


FIG. 1. Section of parotid tumor from a female $(C3Hf/_{Lw} \times AKR)_{F2}$ mouse that had received no X-ray. Mouse received PTA of 10th P388 D_1 tissue culture passage at birth and was autopsied at 5 wk of age. Note poorly-defined tubular structures (upper right) surrounded by and adjacent to round and spindled cells presenting a sarcoma-like appearance, typical of PTA-induced parotid tumor. Hematoxylin and eosin, ×215.

FIG. 2. Section of parotid tumor from a female $C3Hf/_{Bi}$ mouse, thymectomized at one mo of age and irradiated with 300 r to whole body at 6 wk of age. Mouse received PTA of 10th P388 D_1 tissue culture passage 2 days following irradiation. Autopsy was performed when mouse was 5 mo old, at which time it bore a large tumor of the left parotid gland and 2 mammary tumors. Histologically, this tumor did not differ from that illustrated in Fig. 1. Hematoxylin and eosin. ×215.

FIG. 3. Section of thymic epithelial tumor from a female $(C3Hf/_{Lw} \times AKR)_{F2}$ mouse that had received no X-radiation and was inoculated with 10th P388 D_1 tissue culture passage of PTA at birth. Autopsy was performed at one mo of age. Epithelial tumor cells form solid mass at left. Lymphocytes occupy lower right corner of field. Hematoxylin and eosin. ×215.

FIG. 4. Section of thymic epithelial tumor from a female $C3H/_{Bi}$ mouse that had received a transplant of PTA-infected salivary gland from tissue culture, at one mo of age. This animal also received 300 r of total-body X-irradiation on day of transplantation. At autopsy 3 mo later, tumors were found in all salivary glands, exorbital lacrimal glands, thymus, thyroid, hair follicles, and bone. Thymic tumor shown here contained fewer lymphocytes than that in Fig. 3, but was otherwise identical. Hematoxylin and eosin. ×215.

FIG. 5. Section of kidney from same mouse as in Fig. 3. Small inflammatory infiltrates are present about portions of renal tubules that contain enlarged epithelial cells with ''ballooned'' nuclei and intranuclear inclusions. Such changes were very numerous in kidneys of mice inoculated with PTA as newborns. Hematoxylin and eosin. ×215.

FIG. 6. Section of kidney from same mouse as in Fig. 4. No significant changes are present. Lesions of type shown in Fig. 5 were consistently absent from kidneys of mice inoculated with PTA as adults. Hematoxylin and eosin. ×215.

The histologic appearance of primary tumors encountered in Groups 1 and 2, irradiated adult $C3Hf/_{BiLw}$ mice, did not differ significantly from that described earlier for many strains of mice receiving virus as newborns. A direct comparison with tumors induced in non-irradiated mice that received the same virus preparation, but in smaller dose and by subcutaneous route at birth, has limited value because the salivary gland tumors in the irradiated mice were much larger than those in the non-irradiated mice inoculated as newborns. Mice of the latter group reached the stage of terminal illness from thymic tumor effects at a time when their salivary tumors were still only microscopic in size. Nevertheless, the basic tumor pattern, with a marked tendency toward loss of epithelial character and gain of spindle-cell features, was observed in both irradiated and the non-irradiated groups (Fig. 1 and 2). Likewise, tumors of thymus (Fig. 3 and 4), hair follicle, and thyroid found in irradiated mice differed histologically in no way from PTA-induced tumors in these same tissues in non-irradiated mice. Microscopically, no evidence was seen to indicate that X-irradiation had any qualitative effect on the pathogenesis of those tumors that developed.

Quantitatively, however, no tumors were observed in submaxillary, sublingual, and accessory glands of the oropharynx, or in submucosal glands of the nasal passages and trachea in the X-irradiated adult animals. Furthermore, the renal cortical epithelial lesions so characteristic of PTA infection in newborn mice were absent in these adult animals, even when parotid tumors were present (Fig. 5 and 6). The most sensitive histologic indicator for this particular strain of the agent would thus appear to be parotid tumor development.

Table III presents results of tumor response of mice inoculated when less than 24 hours of age with the same virus preparation used in the adult study.

Two mice of the thymectomized group were positive for HI antibodies (HI>200) at time of virus inoculation and have remained tumor-free. All others in this group and in Group 1 (Table I) were HI negative. Sera of the mice in Group 3 were not tested for HI antibodies.

Preliminary results have been reported on transfer of PTA-infected explants of salivary gland tissue to irradiated mice(8). There are several interesting aspects of the completed results (Table IV) of these mice which have now reached 17 months of age. Response of adult irradiated mice receiving 48 day explants of salivary gland tissue from cultures exposed to PTA was similar to that of mice receiving PTA at birth. Bilateral parotid gland tumors appeared early at 1 to 2 months after transfer of cultures, and there were observed primary tumors of other salivary glands, of the lacrimal glands, and of thymus, hair follicles, bone, thyroid gland, mammary glands and subcutaneous connective tissues. In contrast, only 3 of 20 mice receiving cells adherent to the glass of these test cultures, developed parotid tumors. These were unilateral, there was only one other primary site involved in tumor formation, and the latent periods were long. In addition 2 thymic epithelial tumors were observed at 14 and 15 months, respectively, in mice receiving fluid and cells of the test cultures. In one of these mice there was no gross evidence of parotid tumors; histologic sections were not available. In the other, the histologic sections presented no evidence of parotid tumors. A liposarcoma and a bone tumor appearing in mice receiving cells and fluid from cultured explants are probably unrelated to PTA, as they have been seen occasionally in control C3Hf/$_{\mathrm{BiLw}}$ mice, and were histologically distinct from PTA-induced tumors of similar tissue origin.

In only one of the necropsied tumor-bearing mice was there gross or microscopic evidence of a tumor at the site of transfer of explants or tissue culture fluids and cells. This was a fibrosarcoma in the right axilla of a mouse also bearing a unilateral parotid tumor. This mouse had received tissue culture scrapings of cells and fluid. More recently, however, progressively growing neoplasms at site of transplant(9) have been observed following transfer of several cultures of salivary gland tissue to mice in the neonatal period after varying periods of PTA exposure *in vitro*.

One animal in 10 C3Hf/$_{\mathrm{BiLw}}$ mice receiving control explants, developed a unilateral parotid tumor at 10 months of age. This is not surprising in view of the ease of cross-contamination and extreme stability of this virus.

Nine neoplasms (6.5%) of reticular tissues (leukemia) were found among the 139 mice under observation. This frequency is somewhat lower than that previously observed in this strain of mouse(2) but many mice are still alive. These were not found in excess in the mice receiving PTA.

Discussion. The data presented show that tissues of non-immune adult mice of the C3Hf/$_{\mathrm{BiLw}}$ strain are capable of responding to PTA (polyoma virus) by development of neoplasms of the parotid gland. The resistance of adult mice is similar to a strain resistance observed in C57Bl/$_{\mathrm{Ka}}$ mice to PTA (1). Relatively few primary sites are involved in tumor induction and the latent period is long. The striking thymotrophic character of the Passage X P388D$_1$ strain of PTA, as observed in mice receiving virus shortly after birth was not expressed by adult irradiated mice. Thus thymectomy would not be expected to influence the response in adults.

The role irradiation plays in influencing sensitivity of adult tissues needs to be investigated. Additional information obtained in this laboratory shows that:

1) whole body X-irradiation given 3 days to 1 week after introduction of virus is as effective in modifying sensitivity as irradiation given prior to virus, and

2) shielding of a thigh during irradiation does not influence the response.

Despite the fact that infection by polyoma virus occurs spontaneously in our breeding colonies at a frequency greater than 30% in mice over 3 months of age(4), an extremely low incidence of parotid tumors, and at lengthened latent periods has been observed in mice of the following inbred strains receiving fractionated whole-body X-radiation at total doses of 400 to 672 r: $C57Bl/_{Ka}$, 400 mice, 0 parotid tumors; RFM, 80 mice, 0 parotid tumors; $C3Hf/_{Lw}$ 92 mice, 1 parotid tumor; $AKR/_{Lw}$, 165 mice (thymectomized) 2 parotid tumors; $C3Hf/_{BiLw}$, 159 mice, 2 parotid tumors. These strains were observed throughout life.

The likelihood that relative resistance of non-immune adult mice to the tumor-inducing effects of PTA is a dose-response phenomenon is indicated by 2 observations: 1) Irradiated $C3Hf/_{BiLw}$ adults given 48-day PTA-infected submandibular gland explants have revealed a high potential response by developing primary tumors in a variety of sites at short latent periods and in a majority of animals, resembling the response of mice inoculated shortly after birth, and 2) adult submandibular explants infected with PTA *in vitro* appear to be more susceptible to the proliferation-inducing effects of virus than explants of tissue from newborn mice(1). Unfortunately no quantitative data are available at the moment to answer the questions.

It is clear that a peculiar susceptibility of the newborn mouse to PTA is not a prerequisite for induction of tumors as evidenced particularly by the response of those C3H mice, discussed above, that received virus from infected explants of salivary gland tissues.

Summary. Pleomorphic tumors of the parotid gland have been induced by PTA (polyoma virus) in non-immune adult $C3Hf/_{BiLw}$ mice given 300 r whole-body X-radiation. The latent period was long and few other primary sites were involved in tumor induction following intravenous injection of a virulent thymotrophic virus preparation. Thymic epithelial tumors, induced in 100% of mice injected in the neonatal period, did not appear in mice injected as adults. Thymectomy therefore could not be shown to have an influence on the expression of tumors in adult-injected mice. The response of X-rayed, adult $C3Hf/_{BiLw}$ mice receiving salivary gland explants exposed *in vitro* to PTA, was in contrast similar to the response of mice receiving virus within 24 hours *post partum*. Tumors arising at many primary sites in a relatively short period of time were observed. No differences were found in the pathogenesis of the tumors in the groups studied here. The typical renal cortical epithelial lesions characteristic of PTA infection in newborn mice were absent in those animals receiving virus as adults.

The authors are indebted to Mr. Britton Smith and Mr. Willie Morgan for technical assistance and to Mr. Gebhard Gsell for photography.

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The Vacuolating Virus, S.V.$_{40}$ (26128)

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Viruses are commonly carried by monkeys and may appear as contaminants in cell cultures of their tissues, especially the kidney. Included among these are B virus, foamy agent, measles-like virus, hemadsorption viruses, LCM virus, arborviruses, and a wide variety of miscellaneous viral agents(1-7). Hull *et al.*(2,3) have studied extensively viruses encountered in "normal" monkey renal cell cultures. Hull called them simian or SV viruses and classified them into 4 groups based on the kind of cytopathic change induced in monkey kidney cell cultures infected with the agents. Twenty-eight of these viruses were precisely separated serologically into types and, additionally, 24 unidentified viruses were recorded. Malherbe and Harwin(4) distinguished 7 distinct types among the simian agents or SA viruses they recovered from vervet kidney materials.

During the past 2 years, our virus research group* has repeatedly encountered a new simian virus of rhesus and cynomolgus monkey kidney origin. This agent was unique among simian viruses studied hitherto since it grew but did not cause a cytopathic effect in the rhesus or cynomolgus kidney cell cultures from which it was derived. Instead, it grew and caused marked cytopathic changes in cell cultures of a heterologous species, *i.e.*, the green monkey, *Cercopithecus aethiops* obtained from Equatorial East Africa [grivet, according to Sanderson(8)]. This agent has been called the "vacuolating virus" by us because of the prominent cytoplasmic vacuolation seen in infected cell cultures. Dr. Hull† suggested that this agent be given the "official" designation of S.V.$_{40}$ in C.P.E. group 4.

The discovery of this new virus, the vacuolating agent, represents the detection for the first time of a hitherto "non-detectable" simian virus of monkey renal cultures and raises the important question of the existence of other such viruses. The present report summarizes the current information available concerning the vacuolating agent. Certain of these data have been presented at the Second International Conference on Live Poliovirus Vaccines(9).

Materials and methods. Tissue cultures. Primary cell cultures were prepared from trypsinized cell suspensions and were grown in Melnick's lactalbumin-yeast extract medium(10) containing 2% heat-inactivated calf serum. All line cell cultures were grown in Eagle's basal medium(10) containing 10% heat-inactivated horse serum. During virus propagation, all cell cultures were maintained using medium 199(10) containing 2% heat-inactivated chicken serum and at initial pH 7.6. All culture fluids contained 100 μg of penicillin and 100 units of streptomycin per ml.

Propagation of vacuolating agent. The virus grew readily in cell cultures of grivet monkey kidney with production of cytopathic change. Such cultures were used routinely for the work described in this report. For ordinary purpose, the virus was passed in an initial dilution of 10^{-1} of infected tissue culture fluid using 0.2 ml of inoculum per 2.0 ml of maintenance medium. After 4 to 7 days incubation, at a time when at least 50% of the cell sheet showed cytopathic change typical of that of the vacuolating agent, the cultures were frozen and thawed once and the fluid was harvested. The virus was stored frozen in the dry ice refrigerator.

Virus titrations and serum neutralization tests. All virus titrations were performed using serial 10-fold dilutions of virus. The inoculum dose was 0.2 ml per roller tube culture containing 2.0 ml of maintenance medium. The end point was the highest initial dilution of virus which caused degeneration of 25% or more of the cells in the cultures after 7 to 10 days incubation. In the neutralization tests, serial 2-fold dilutions of serum inactivated at 56°C for 30 minutes were incubated for one hour at 37°C with an equal volume of virus diluted to contain about 200 TCD$_{50}$ per 0.2 ml. The inoculum was 0.2 ml of the virus-serum mixture and the end point was the highest initial dilution of serum which effected complete suppression of cytopathic change in cultures observed 7 to 10 days following inoculation.

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* The first such agent was recognized by Dr. L. McClelland in the course of safety testing of vaccines.

† Personal communication.

Preparation of antiserum against the vacuolating agent. Young adult rabbits were injected intravenously with 1.0 or 2.0 ml of infected grivet monkey kidney culture fluid plus 1.0 ml of infected fluid emulsified in Arlacel-Drakeol adjuvant administered intramuscularly in a single dose or intracutaneously in divided doses. A final 1.0 ml dose of infected fluid was administered intravenously 3 weeks later and the animals were bled one week thereafter.

Results. Recovery of strains of vacuolating virus. Vacuolating virus was recovered from "normal" cell cultures of rhesus and cynomolgus monkey kidney and from the seed stocks of a variety of viruses which had become contaminated with the vacuolating virus during passage in cultures of rhesus and cynomolgus monkey kidney.

The vacuolating virus was recovered from contaminated virus seeds by passage of the virus seed, in the presence of its homologous antiserum, in cell cultures of *Cercopithecus aethiops* or grivet monkey. Under these conditions, the vacuolating virus "broke through" and was identified in serum neutralization tests using vacuolating virus antiserum prepared in rabbits. Recovery of the virus from "normal" rhesus or cynomolgus cell cultures was accomplished by simple passage, in grivet cell cultures, of the fluid from the rhesus or cynomolgus cultures which had been held for 7 to 10 days with maintenance medium. Vacuolating virus isolates were identified in serum neutralization tests using rabbit antisera prepared against prototype strains.

TABLE I. Origin of 8 Strains of Vacuolating Virus.

Strain	Recovered from	Infectivity titer of original material
776	Adenovirus type 1, seed stock	N.D.*
175	" " 5, " "	"
S 207	Sabin, type 1, live attenuated oral polio vaccine, strain LSC	$10^{-4.5}$
S 211	Sabin, type 2, live attenuated oral polio vaccine, strain P712	$10^{-3.7}$
S 215	Sabin, type 3, live attenuated oral polio vaccine, strain Leon	$10^{-4.5}$
953	Uninfected "normal" rhesus monkey kidney cell culture	$10^{-5.5}$
1095	*Idem*	$10^{-6.0}$
584	Uninfected "normal" cynomolgus monkey kidney cell culture	$10^{-5.5}$

* Not done.

More than 20 strains of vacuolating virus have been recovered in our laboratory. Table I shows the origin of 8 of the strains examined most extensively. The vacuolating virus has appeared as a contaminant in the seed stocks of all of types 1 through 7 adenovirus. These seeds had been prepared in cultures of rhesus or cynomolgus monkey kidney and were obtained from Dr. R. J. Huebner or were brought to this laboratory by one of us (M.R.H.) from Walter Reed Army Inst. of Research. Additionally, vacuolating virus was recovered from seed stocks of *Myxovirus parainfluenza* 1 and 3, from the SA virus, and from the respiratory syncitial agent received from Dr. R. Chanock and Dr. K. Habel.

Vacuolating virus strains S207, S211 and S215 were derived from types 1, 2 and 3, respectively, of Dr. Albert Sabin's live attenuated poliomyelitis vaccine. For recovery of vacuolating agent, the poliovirus was neutralized by homologous polio immune serum prepared in rabbits immunized with Syverton's HeLa cell lines of poliovirus propagated in human stable amnion cell culture. The infectivity titer of the vacuolating virus in each of the Sabin vaccines ranged from $10^{-3.7}$ to $10^{-4.5}$. These isolates of vacuolating virus were identified in serum neutralization tests with rabbit antisera against prototype strain

776. At the time of the initial isolations of vacuolating virus, the Sabin vaccines were also passaged in presence of a mixture of homologous poliovirus and vacuolating agent antiserum. The antiserum mixtures suppressed both the poliovirus and vacuolating agent and showed the absence of other detectable viruses which might have been present in the Sabin materials.

The essentially ubiquitous occurrence of vacuolating virus in the various virus seed stocks and vaccines suggested a high infection rate among normal rhesus and cynomolgus monkey kidney cell cultures. This was borne out in tests for virus in "normal" cell cultures. During a one-month period, 10 rhesus and 10 cynomolgus monkey kidney cell lots prepared for use in ordinary Salk vaccine production were examined for presence of the vacuolating virus by passage to grivet kidney cultures. Each lot of monkey kidney was derived from a pool of kidneys from 2 or 3 monkeys. None of these "normal" cell lots presented any cytopathic change suggestive of the vacuolating virus. Of 10 lots of rhesus kidney, 7 yielded vacuolating virus. One lot was infected with a foamy-like virus(1) and 2 lots appeared free of simian contaminants. Only one of the 10 cynomolgus cell culture lots revealed vacuolating virus. However, it was not possible to carry out definitive tests of these materials since 8 of the lots were heavily contaminated with what appeared to be foamy virus. Thus, the vacuolating agent, if present, may have been overgrown and excluded by the foamy virus.

It is striking that the titer of vacuolating agent was as high as $10^{-6.0}$ in the fluids from the normal rhesus kidney cell cultures, even though these cultures showed no cytopathic change suggestive of the presence of a virus.

Cultures of grivet monkey kidney prepared in our laboratories have proved remarkably free of the vacuolating agent. During use of more than 1000 lots of individual monkey kidney cell cultures, presence of the vacuolating agent was suspect in only 4 lots and proved for only 2 lots.

Properties of vacuolating virus. Cytopathic effect. Fig. 1 shows the outstanding cytopathic changes caused by vacuolating virus in grivet kidney as observed in wet and in stained culture preparations. Grivet kidney cell cultures infected with about 1000 TCD_{50} of vacuolating virus show beginning cytopathic changes on day 3 or 4, when some of the cells may appear rounded or shrunken and the cytoplasm may be darkened. A few cells may show beginning vacuolation of the cytoplasm. With increased time, typical changes develop, consisting predominantly of ballooning of cells which are free or spread out on the glass together with intense vacuolation of the cytoplasm of such cells. These vacuoles are highly refractile in wet preparations and appear as "holes" with intensely stained boundaries in hematoxylin and eosin stained materials. In cultures stained with acridine orange and observed by ultraviolet fluorescent microscopy, the vacuoles likewise appear to be "holes" in the cytoplasm. We have not been able to demonstrate any stainable substance within the vacuoles nor has it been possible to demonstrate inclusion bodies

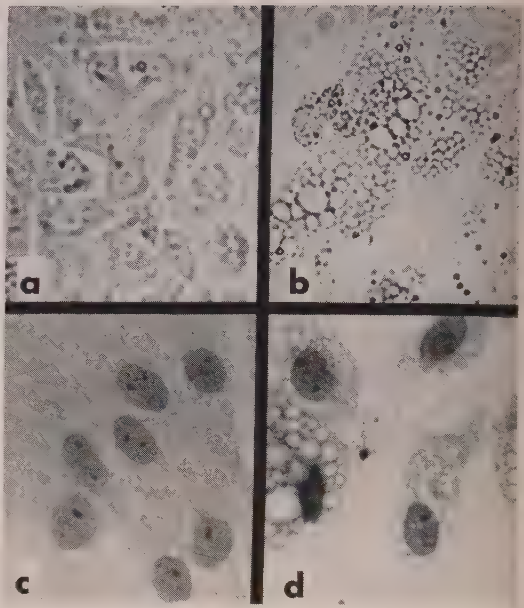

FIG. 1. Cytopathic changes in infected kidney cell cultures of *Cercopithecus aethiops* monkeys caused by strain 776 of vacuolating virus. a. Uninoculated control culture, unstained, day 6, 188 × mag. b. Infected culture, unstained, day 6, 188 × mag. c. Uninoculated control culture, day 6, 442 × mag., stained with H & E. d. Infected culture, day 6, 442 × mag., stained with H & E.

TABLE II. Antigenic Relationships of Vacuolating Virus Strains as Measured in Serum Neutralization Tests.

Virus	Serum titer	
	776	175
776	1:4096 or >	1:800
175	1:4096 or >	1:1600
1095	1:4096 or >	1:2048
953	1:4096 or >	1:4096 or >
584	1:4096 or >	1:4096

in such infected cells. Nuclei of infected cells may appear normal or disorganized internally. Limited fusion of the cytoplasm of adjoining infected cells may occur but, predominantly, each cell maintains its own integrity. Thus, the syncitium or giant cell formation, so characteristic of measles, foamy virus and certain other virus infections, is not prominent in vacuolating virus infection and this serves as a distinguishing feature. Terminal cytopathic changes occur in the cultures 5 to 10 days post-inoculation when the infected cells aggregate together and detach from the glass leaving behind only small islands of normal or degenerated cells. The cytopathic changes caused by vacuolating virus appear to be quite distinct from those described for Hull's 4 C.P.E. groups of simian viruses or for Malherbe's and Harwin's 7 simian agents.

Antigenic relationships. All strains of vacuolating virus studied to date have comprised a single immunologic group. Antisera against vacuolating virus strains 776 and 175 neutralized the homologous viruses and all of 3 additional strains as well (Table II). Additionally, and not shown in the table, all 3 of the Sabin vaccine isolates (S207, S211, S215) were neutralized by these sera.

Strain 776 of vacuolating virus was not neutralized by antiserum against C.P.E. group 4 strain S.V.$_{6}$, S.V.$_{26}$ or S.V.$_{29}$ antisera furnished by Dr. Hull. Additionally, it was not neutralized by Myxovirus parainfluenza 1 or 3 antisera or by such SA, S.V.$_{5}$ or foamy virus antisera as were available to us. Vacuolating virus strains 776, 175, 953, 1095 and 584 were neutralized by pooled antisera against Hull's group 1B (S.V.$_{20,\ 23,\ 25,\ 27}$) and the last three strains by group 3 (S.V.$_{12,\ 28,\ 59}$). None was neutralized by group 2 (S.V.$_{2,\ 16,\ 18,\ 19}$) or by group 4 (S.V.$_{5,\ 6,\ 29}$) antisera. In these tests, only a negative finding had significance since the antisera employed were prepared using virus grown in rhesus or cynomolgus monkey kidney cell cultures and these might also have contained vacuolating virus.

Host range. A preparation of vacuolating virus strain 776 which titered 10^{-5} in grivet kidney culture was tested in the various primary and line cell cultures shown in Table III. The virus was cytopathic for patas kidney and for rhesus monkey testicle, but these cells were about 30 to 300 times less sensitive than grivet kidney. Vervet monkey kidney has also been reported highly susceptible to the vacuolating virus(11). Grivet and vervet monkeys are separate races of the species *Cercopithecus aethiops*.

No definitive cytopathic change referrable to the vacuolating virus was observed in any of the remaining primary or line cell cultures, shown in the table, when observed for eight to twelve days. The virus persisted for at least 7 days in most of the cultures without increase in titer. Limited proliferation might have occurred. Titers as high as 10$^{-8.5}$ have been obtained with vacuolating virus grown in grivet kidney.

The susceptibility of suckling mice and of embryonated hen's eggs to the vacuolating virus was tested. Suckling mice, inoculated intracerebrally, subcutaneously or intraperitoneally failed to develop illness within 21

TABLE III. Titration for Cytopathic Effect of Vacuolating Virus Strain 776 in Various Cell Cultures.

Kind of cell	Titer based on cytopathic end point, 8 days
Primary cell cultures	
Grivet monkey kidney	$10^{-4.5}$
Patas ” ”	$10^{-3.0}$
Rhesus ” testicle	$10^{-2.0}$
Rhesus ” kidney	$<10^{0}$
Rabbit kidney	$<10^{0}$
Human amnion	$<10^{0}$
Line cell cultures	
HeLa	$<10^{0}$
Stable amnion	$<10^{0}$
Chang liver	$<10^{0}$
Girardi human heart	$<10^{0}$

TABLE IV. Filterability of Vacuolating Virus Strain 776.

Filter used	Infectivity titer of filtrate
Seitz, S1	$10^{-3.75}$
Selas 03 candle	$10^{-3.25}$
Unfiltered, control	$10^{-5.00}$

TABLE V. Heat Stability at 56°C, 1 Hour, of Infectivity of Vacuolating Virus Strain 776.

Virus menstruum	Infectivity titer
Medium 199	
Heated 1 hr, 56°C	$10^{-4.0}$
Not heated	$10^{-5.5}$
Medium 199 with 2% normal rabbit serum	
Heated 1 hr, 56°C	$10^{-3.75}$
Not heated	$10^{-5.5}$

days following primary inoculation of vacuolating virus or following subpassage of brain or carcass carried out 7 days following primary inoculation. Embryonated hen's eggs inoculated after 8 days incubation *via* the allantoic or amniotic routes failed to develop manifest signs of disease within 6 days primary incubation. Additionally, none of the harvested fluids contained demonstrable hemagglutinins for chick erythrocytes.

Miscellaneous physical and biological properties of the vacuolating virus. The vacuolating agent was readily filtered through bacterial sterilizing filters (Table IV). The agent is relatively heat stable compared with most viral agents (Table V). Fig. 2 presents data relating to inactivation‡ at 37°C with 1:4000 formalin of the vacuolating agent. The slope of the inactivation curve indicates a slightly less rapid inactivation rate than that ordinarily found for poliovirus under the same conditions. Table VI presents a summary of known biological and physical properties of the vacuolating agent.

Occurrence of antibody against vacuolating virus in human and monkey sera. Table VII summarizes the results of tests for antibody against the vacuolating virus in sera of human subjects and in normal monkey sera. None of the normal or pre-vaccination sera

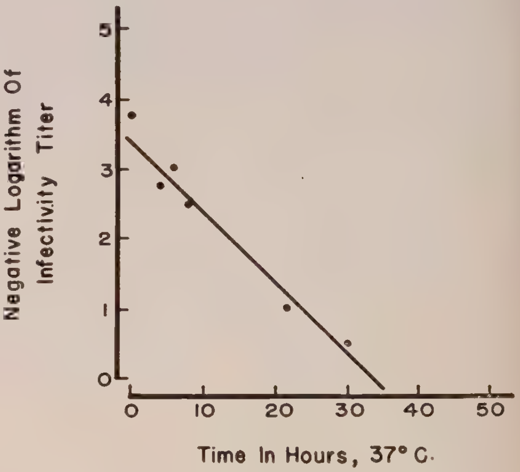

FIG. 2. Inactivation kinetics, vacuolating virus strain 776 with 1:4000 formalin at 37°C.

TABLE VI. Summary of Biological and Physical Properties of Vacuolating Virus.

1. Widespread "inapparent" virus in normal rhesus and cynomolgus kidney cell cultures. May also occur as an "apparent" contaminant of grivet kidney cell cultures.
2. Cytopathic for grivet kidney, vervet kidney, patas kidney and rhesus testicular cells in culture. Fails to cause cytopathology in a variety of other cells in culture.
3. Fails to cause manifest illness or death in newborn mice or in embryonated hen's eggs.
4. Known strains comprise a single serotype.
5. Filterable through Seitz S1 and Selas 03 filters.
6. Infected cultures or culture fluids fail to hemadsorb or to cause hemagglutination of guinea pig, chicken, or human "O" erythrocytes at 4°C or at 25°C.
7. Resists treatment for 18 hr with an equal volume of diethyl ether.
8. Infectivity destroyed by treating with 1:4000 formalin at 37°C.
9. Relatively heat stable. Heating at 56°C for 1 hr reduces infectivity titer about 30-fold.
10. Stable on storage at –20°C and at –70°C.
11. Induces homologous neutralizing antibody in rabbits immunized with the agent.
12. Sensitive to photodynamic inactivation with visible light in presence of certain dyes(14).
13. Preliminary findings indicate that agent is infectious for man when given by respiratory route(14).

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‡ We are indebted to E. Dwyer for these data.

TABLE VII. Tests for Neutralizing Antibody against Vacuolating Virus Strain 776 in Various Human and Monkey Sera.

Description of sera	No. positive / No. tested
Military recruits:	
Received 2 doses of killed adenovirus vaccine	
Pre-vaccination sera (diluted 1:5)	0/9
Post-vaccination sera (diluted 1:5 and 1:20)	9/9 *
Received 2 doses of placebo	
Pre-injection sera (diluted 1:5)	0/7
Post-injection sera (diluted 1:5)	0/7
Children:	
Received 2 doses of Salk poliomyelitis vaccine	
Lot 43038—Pre-vaccination sera (diluted 1:5)	0/7
Post-vaccination sera (diluted 1:5 and 1:20)	5/7 †
Lot 43058—Pre-vaccination sera (diluted 1:5)	0/6
Post-vaccination sera (diluted 1:5)	1/6
Fed Sabin live oral polio vaccine on 6 occasions, serum diluted 1:5	0/5 ‡
Normal children, not fed Sabin vaccine	0/5 ‡
Human gamma globulin, Philadelphia Serum Exchange, lot G50B, diluted 1:50	0/1
Normal grivet monkey sera (5 lots) (undiluted and 1:5)	0/14
Normal rhesus monkey sera (diluted 1:5)	12/18

* 8/9 titered 1:20 or >. † 3/7 titered 1:20 or >. ‡ Sera tested for Dr. A. Sabin.

from any of the human subjects contained antibody against the vacuolating virus nor was such antibody present in a lot of human gamma globulin. This suggests that natural infection in man with the vacuolating virus, if it occurs at all, is infrequent. However, all 9 persons given 2 doses of formalin-killed adenovirus vaccine prepared in rhesus or cynomolgus monkey kidney cell culture by one of us (M.R.H.) at the Walter Reed Army Inst. of Research(13), developed antibody against the vacuolating virus. Similarly, 7 of 13 persons given 2 doses of Salk poliomyelitis vaccine developed antibody against the vacuolating virus. These results are wholly consistent with the high frequency of vacuolating virus in monkey renal cell cultures, and indicate also the apparent high potency of vacuolating virus antigen in these vaccine preparations. None of 5 persons developed antibody against the vacuolating virus when fed Sabin live polio vaccine on 6 occasions. These data and those obtained more recently by Sabin(14) suggest lack of infectiousness of the vacuolating virus when administered by the oral route. This is in contrast to the apparent ability to infect man when the virus was administered by the respiratory route (12).

None of 14 normal sera from grivets representing 5 different shipments contained antibody against the vacuolating virus. By contrast, 12 of 18 sera from rhesus monkeys proved to have antibody to the virus. These findings are consistent with the very infrequent finding of vacuolating virus in grivet kidney cultures and the high prevalence in rhesus kidney.

Discussion. The discovery of the vacuolating virus represents the first demonstration of a hitherto hypothetical "non-detectable" simian virus, *i.e.,* a virus present in monkey kidney cell cultures but not detectable by current procedures. All of the simian viruses which were recovered previously and which were of monkey kidney origin were detected in kidney cell cultures of the same monkey species, either in the primary culture itself or on sub-passage. In the present instance, the vacuolating virus was detected only following passage in the kidney cell cultures of a heterologous host. The demonstration of this first "non-detectable" simian virus raises the question of whether other as yet undetected viruses might also await demonstration. Such experience, although new to simian viruses and monkey kidney, is neither new nor unique to other viruses and cell culture systems.

The discovery of the vacuolating virus has special significance during the contemporary period in relation to live poliovirus vaccine. As shown in this report, all 3 types of Sabin's live poliovirus vaccine, now fed to millions of persons of all ages, were contaminated with vacuolating virus.

It is well established that when 2 different viruses compete in the same cell culture or animal host system, the end result may be interference with growth of one or both of the viruses, enhancement of pathogenic effect, or no immediate demonstrable effect. The interference effect is documented in numerous publications(15). The recent demonstration by Dalldorf and Weigand(16) that monkeys may become paralyzed following cerebral injection of both Coxsackie A14 and live attenuated type 1 poliovirus but not following either alone, serves as a cogent example of mutual potentiation of pathology by 2 viruses in consort.

The presence of the contaminating vacuolating virus in such live poliovirus vaccine raises the question, at least, of the validity of tests for monkey neurovirulence (*cf.* 17) following cerebral, spinal or muscular inoculation(18,19) and of the tests for "genetic" markers of poliovirus(20) such as the t, MS, d and others carried out in tissue culture and subject to such competitive effect. The question may also be raised whether observed alterations in neurovirulence and other "genetic" marker properties attending passage of live attenuated poliovirus through the human gut might reflect, in part at least, alterations in the poliovirus-vacuolating virus ratio in such materials submitted for test.

The essentially ubiquitous occurrence of vacuolating virus in seed stocks of a multiplicity of the "newer" respiratory viruses, as recorded herein, raises the question of the possible influence of vacuolating virus on the outcome of tests carried out in human volunteers to determine the pathogenicity of these respiratory agents for man. Similarly, the possible influence of vacuolating virus on results of diagnostic CF tests employing antigens prepared in rhesus or cynomolgus monkey kidney requires review.

The failure, to date, to detect excreted vacuolating virus or development of antibody against the agent among persons fed live poliovirus vaccine(14) strongly suggests lack of massive proliferation of the virus in the human intestinal tract under the conditions employed although possible limited proliferation under these circumstances cannot be excluded. Alternatively, demonstration of antibody development among persons given vacuolating virus *via* the respiratory tract(12) provides strong evidence for infectivity of the virus in man when given by this route. The lack of significant harmful effect in man, in the short term, is well established in results of studies in millions of volunteers fed to date. Less can be said concerning a possible long-term effect. For example, the highly theoretical question has already been raised (21) concerning the possibility of oncogenic properties of viruses of unknown quality, such as the vacuolating virus, especially when administered to babies. Induction of tumors in hamsters following infection by parenteral or extraparenteral routes, early in life with polyoma-infected mouse tissue(22,23) presents an experimental analogy for the hypothetical situation in man. While such possibility is only theoretical and certainly remote, the determination must await the outcome of the experiments which may be decades in process. The argument(24) that human subjects continually consume viruses in foods with no apparent ill effect is lacking in substance since raw monkey kidney is not ordinarily part of the human diet.

In the practical sense, the vacuolating agent appears to be just "one more" of the troublesome viruses to be conquered in the quest for vaccines which are safe and effective when used in man. For preparing killed vaccines as, *e.g.*, Salk poliomyelitis vaccine, the matter is simply dealt with by virtue of the susceptibility of the vacuolating virus to the inactivating action of formaldehyde. For live virus vaccines, the simple solution seems to involve elimination of the agent from the seed stocks and to discard any contaminated lots of the material.

Summary. The newly discovered vacuolating virus, $S.V._{40}$, appears to be a common and essentially ubiquitous contaminant of rhesus monkey kidney cell cultures and a likely common contaminant of cynomolgus kidney cultures in which it develops to high titer without evident cytopathic change. The virus grows readily in grivet, vervet and patas kidney and in rhesus testicle cultures causing a unique cytopathology characterized by ballooning and intense vacuolation. Some biological and biophysical properties of the virus are described. Principal interest in and importance of the vacuolating virus arise from its demonstrated presence in all 3 types of Sabin's live poliovirus vaccines and in numerous respiratory virus seed lines employed in human volunteer inoculation experiments. Demonstration of the virus has raised the question of safety following administration to human subjects, especially infants, and of possible lack of validity of conclusions reached from experiments to measure neurovirulence or "genetic" markers of attenuated poliovirus vaccine strains and to determine the pathogenicity of respiratory viruses for man. The vacuolating virus is effectively dealt with in killed virus vaccines by virtue of its susceptibility to formalin. The optimal solution to the live virus vaccine problem appears to lie in elimination of the virus from such preparations.

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Received Sept. 26, 1960. P.S.E.B.M., 1960, v105.

Infertility in Rabbits Induced by Feeding Ladino Clover.* (26129)

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It is now well established that potent estrogenic activity is extractable from a variety of commonly-used forage crops(1,2). Bennetts (3) describes a number of severe abnormalities of the reproductive tract of sheep pastured on Dwalganup strain of subterranean clover, and it is assumed that derangements of reproductive performance were caused by high estrogenic content of the clover (10 μg/100 g dry weight) because effects similar to those produced by injection of estradiol were duplicated by feeding the same strain of clover to guinea pigs(4). On the other hand, Kendall *et al.*(5) felt that partial infertility in New Zealand rabbits accompanied by hemorrhaging, numerous abortions and resorptions, and reduction in litter size, was due to a nutritional imbalance caused by feeding a diet of 50% soybean hay.

The research reported here was prompted by reports from several dairy farmers in New Hampshire of reproductive failure of heifers and dairy cows fed on a diet rich in Ladino clover. In each instance substitution of a timothy hay diet quickly corrected infertility. Interestingly, dairymen from other areas in the state, and in Mass. and N. J., indicated no reproductive problem on feeding Ladino. Nevertheless, occurrences of infertility seemed widespread enough to warrant at least pilot experimentation. Rabbits were selected as test animals with the thought that endocrine disturbances of reproductive processes induced by Ladino feeding, if any, could be worked out less expensively, in a shorter time, and in less space than if dairy animals were used.

Materials and methods. Domestic rabbits, principally of New Zealand and Flemish giant strains, 5-12 months old, were maintained in a well-ventilated room equipped with automatically-timed lighting from 7 a.m. to 9 p.m. throughout the year and on a diet of water and commercial rabbit pellets, *ad lib.* Fresh Ladino clover, in season, or Ladino hay (both grown on a University plot at Northwood, N. H.) was substituted for pellets in experimental animals. Extent of Ladino feeding for each test is given in Table I. Nutritive values of rabbit pellets and a diet of Ladino clover were virtually identical in important areas of crude protein, digestible protein, crude fat, ash, and N.F.E. content. Any animal showing signs of ill health was discarded and its data eliminated from final tallies. Three bucks of proven fertility were used for random matings, but never more than one mating was allowed per day. Both experimental and control does were permitted only one mating for any one experiment. Gestation periods were 30-34 days, and there were no known abortions. Hemorrhage, paralysis, or other signs of deficient nutrition were not observed. All does appeared to enter a span of anestrus extending from mid-November to early January; all experiments were consequently confined to the period 15 January to 15 November. Except for the short time mentioned, laboratory conditions induced, or at least did not prevent, continuous estrus in all does, so that they were available for experimentation at any time.

Results. Prefeeding and continuation of Ladino feeding during the supposed gestation period (Groups II, III, Table I) resulted in total infertility. When these animals were returned to a pellet diet 35 days after original mating (56 days after start of Ladino diet) all refused to mate and required 3-36 (avg. 24) days before estrus reappeared. In contrast it should be noted that does mated readily after 3 weeks of Ladino prefeeding earlier in the same experiments. If Ladino feeding was restricted to either prefeeding or

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* Published with approval of Director of N. H. Agric. Exp. Station as Scientific Contribution No. 257. Supported in part by Regional Research Funds under NE-41, Endocrine Factors Affecting Reproduction and Lactation in Dairy Cattle. The author acknowledges with thanks technical assistance of P. J. Hannaway, N. R. Marquis, and W. W. Leavitt.

TABLE I. Effect of Ladino Feeding on Reproductive Performance of Female Rabbits.

Group	No. of animals	Diet prior to mating	Diet from day of mating	No. animals littering	Avg No. in litter
I (control)	20	Pellets	Pellets	18	8.1
II	6	Ladino clover (3 wk)	Ladino clover	0	0
III	"	Ladino hay (2½ wk)	Ladino hay	"	"
IV	"	Pellets	" "	2	5.5
V	"	"	Pellets for 96 hr, then Ladino hay	3	7.7
VI	"	Ladino hay (2½ wk)	Pellets	3	7.0
VII	5	*Idem*	Ladino hay for 96 hr, then pellets	3	8.3
VIII	4	"	Ladino hay	—	0 *
IX	"	Pellets	Pellets	(4)	7.2*

* Implantation sites or embryos in laparotomized animals.

a part or all of the gestation period, fertility was not so seriously affected (groups IV-VII), but on the basis of control performance there was definite impairment of reproduction. All experimental animals which failed to litter were subsequently shown to be normally fertile after return to a pellet diet and a short period of rest. One animal in each of groups VIII and IX was sacrificed at 5, 10, 15, and 20 days following mating and exploratory laparotomies performed. No blood points, implantation sites, embryos, or abnormalities of reproductive tract were found in any of the Ladino-fed animals (VIII), and only one corpus luteum was discovered (on one ovary of the 15-day animal). All pellet-fed rabbits (IX) appeared to be carrying out gestation normally.

Ladino clover and hay were assayed using a 22-day-old rat test, measuring increase in uterine weight 48 hours after the start of twice-daily injections. Samples contained between 25 and 35 μg of estrogenic activity (expressed as diethylstilbestrol) per 100 g oven-dried forage. Rabbit pellets did not contain measurable estrogenic activity. Average daily consumption of pellets or clover per animal was approximately 190 g oven-dried weight. This means that rabbits on Ladino may have received as much as 55-65 μg per day of diethylstilbestrol equivalent orally.

Discussion. There is at present no obvious explanation for Ladino's action on rabbits. Ladino appeared to have an even greater estrogenic content than Dwalganup subterranean clover tested by Curnow *et al.*(4), but use of different bioassays may mean that estrogenic levels reported are not wholly comparable. When Ladino was fed (about 90 g daily) to newly-weaned rabbits for 2-4 weeks there was no premature stimulation of the reproductive tract over that of pellet-fed littermate controls. These young rabbits ingested circa 30 μg daily of diethylstilbestrol equivalent as contained in Ladino, yet they were unaffected, indicating that in spite of the high estrogen level of Ladino as ascertained by injection assay in rats, physiologically inactive amounts were apparently absorbed when given rabbits orally. The question is immediately raised whether the depressing action of Ladino on fertility of rabbits may be entirely unrelated to its estrogenic content.

Laparotomies of Ladino-fed (group VIII) animals indicated that ovulation had not occurred. Failure of ovulation could be an explanation for infertility in those animals pretreated with Ladino before mating but probably not for those which had no Ladino previous to mating, unless the effect of Ladino is so rapid as to exert some influence in the 10-hour interval elapsing between mating and supposed ovulation time (as in group IV). Results of all experiments could be explained on the basis that Ladino interfered with either ovulation or implantation. It is interesting that Ladino silage, obtained from the same Northwood plot, did not adversely affect conception rate of an experimental group of Holstein heifers.

Summary. Feeding Ladino to female rabbits impaired reproductive performance, infertility being complete when clover was fed both prior to and during gestation periods. Cause is unknown but failure of ovulation and implantation was observed, and evidence indicates that infertility may be unrelated to estrogenic content of the clover.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Studies on Binding of Tetracyclines by Dog and Human Plasma.* (26130)

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Several investigators have studied the binding of tetracyclines by dog and human plasma. Binding of chlortetracycline in man has been reported by Sirota and Saltzman(1), Kunin *et al.*(2) and Kivman(3). Kunin *et al.*(2) have studied binding of demethylchlortetracycline in man, and Kohn *et al.*(4) in dog. The extent of protein binding of tetracycline by human plasma was investigated by Kunin *et al.*(2) and Kivman(3), and in dog plasma by Pindell *et al.*(5) and Kohn *et al.*(4). Not all the investigators agreed in their findings and different values were reported for one specific antibiotic and species.

The present paper deals with a comparative study of binding of demethylchlortetracycline with chlortetracycline and tetracycline by dog and human plasma. Extent of protein binding was determined by the dialysis equilibrium method, at drug concentrations approaching therapeutic levels and under physiological conditions of temperature and pH. The effect of temperature and pH on binding of tetracycline was also studied.

Method. The experiments were performed with a pool of dog plasma† (5 males and 6 females) and a pool of human plasma† (4 healthy males). Concentration of protein in plasma was determined by micro-Kjeldahl analyses. Distribution of plasma proteins was determined by free electrophoresis in the Spinco Model H electrophoresis apparatus.‡ Tetracyclines used were radioisotopically labeled as follows: 7-tritio-tetracycline hydrochloride, C^{14} demethylchlortetracycline hydrochloride and C^{14} chlortetracycline hydrochloride. Corresponding specific radioactivities were 13, 0.43 and 0.18 μc/mg. The purity of these tetracyclines, checked by various procedures including microbiological assay, spectrophotometric assay and paper chromatography, was 90% for demethylchlortetracycline hydrochloride, 95% for chlortetracycline hydrochloride and 100% for tetracycline hydrochloride. The technic followed for dialysis was developed by Roepke *et al.*(6). Small volumes (0.005-0.040 ml) of aqueous solutions of the tetracyclines were added to the plasma or to the buffer from a micro syringe-burette.§

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* The author wishes to express her appreciation to Dr. R. R. Roepke for encouragement and guidance, to Mr. R. Kelly for direction in radiometric assay, to Dr. J. H. Boothe, Dr. D. A. Buyske, Dr. J. McCormick and Dr. P. A. Miller for preparation of radioisotopically labeled tetracyclines, to Dr. W. Sweeney for obtaining human plasma and to Mr. N. Strippoli for excellent technical assistance.

† Obtained from heparinized blood centrifuged with a layer of oil.

‡ Determined by Mr. M. C. Davies, Biochem. Research Dept.

§ Manufactured by California Laboratory Equipment Co.

TABLE I. Plasma Protein Distribution by Electrophoretic Separation.

Sample	Albumin	Globulins α_1	α_2	β	γ	Fibrinogen	Total protein, g/100 ml*
	%						
Pool of human plasma	57	4	10	11	12	6	8.6
" " dog "	48	5	7	13	18	9	6.1

* Determined by micro-Kjeldahl analyses.

A cellophane bag (7.6 cm long) of Visking Nojax 18/32 casing was used for dialyzing 1.5 ml of plasma. The bag contained a glass rod (7.6 cm long, 8 mm in diameter) which spread the plasma over a large area. The bag was sealed with a rubber band and placed in a ground glass stoppered test tube containing 10 ml of a solution of buffered saline (0.15M sodium chloride and 0.02M phosphate).‖ Unless otherwise stated the tube was rotated (at an angle approximately 12 degrees from horizontal axis of rotation) at 8 rpm for 4 hours inside an oven kept at 37°-38°C. Dialysis experiments lasting for 2, 4, 6 and 8 hours showed that equilibrium was attained between 2 to 4 hours. Final pH of the plasma and buffer measured at 37°C with a Cambridge pH meter was found to vary from 7.3 to 7.4. After equilibration, concentrations of tetracyclines in plasma and buffer were determined by a radiometric assay using liquid-scintillation counting as described by Takesue *et al.*(7). Degree of protein binding expressed as per cent bound of total drug in the plasma was calculated as follows:

$$\mu\text{g unbound drug/ml plasma} = \mu\text{g drug/ml buffer} \times \frac{\text{g water/ml plasma}}{\text{g water/ml buffer}}.$$

$$\%\ \text{bound} = \frac{(\mu\text{g total drug/ml plasma} - \mu\text{g unbound drug/ml plasma})}{\mu\text{g total drug/ml plasma}}\ 100.$$

Water content of plasma and buffer was determined by drying to constant weight. An average value of 0.948 was obtained for the ratio of water content of plasma to water content of buffer.

Since the same results were obtained for binding of tetracyclines with fresh and frozen plasma, the pools of plasma were preserved by storing them at –26°C.

Results. Separation of plasma proteins by electrophoresis (Table I) showed that the pools of plasma had a normal protein distribution.

Degree of binding of 3 tetracyclines by dog plasma proteins is summarized in Table II. The data show that over the range of concentrations covered (1-10 μg/ml) there was no effect of drug concentration on per cent of drug bound. Similar conclusions were derived from the data obtained with human plasma (Table III).

When pH was varied from 6.7 to 8.1 there was an appreciable increase in binding of tetracycline with increase in alkalinity (Fig. 1).

The effect of temperature on the equili-

TABLE II. Binding of Tetracyclines at Various Concentrations in Dog Plasma.

Drug	Drug conc. in plasma, μg/ml Total	Unbound*	% bound
Tetracycline	.94	.61	35†
	.86	.56	35
	2.66	1.58	40
	2.42	1.61	33
	6.48	4.30	34†
	7.10	4.40	38
			36 ± 3‡
Demethylchlor-tetracycline	1.10	.56	49†
	1.09	.56	48
	2.80	1.41	50
	2.87	1.43	50
	8.69	4.38	50†
	8.13	4.58	44
			48 ± 2
Chlortetracycline	1.20	.38	68†
	1.20	.49	59
	2.79	1.33	52†
	3.56	1.49	58
	9.77	4.30	56†
	9.97	4.19	58
			58 ± 5

* $\frac{\text{g water/ml plasma}}{\text{g water/ml buffer}} \times \mu\text{g drug/ml buffer} = \mu\text{g}$ unbound drug/ml plasma.

† Drug was added to buffer except in these determinations where it was added to plasma.

‡ Mean ± S.D.

‖ KH_2PO_4-NaOH buffer system.

brium was studied with tetracycline. When the dialysis equilibration was performed at 37-38°C, an average¶ value of 36% bound (33-40) was obtained. Slightly lower results, 30% bound¶(27-33), were obtained at 2-5°C. The pH for this latter experiment was 7.5 at 2°C.

Discussion. The data reported here show that the tetracyclines (chlortetracycline, demethylchlortetracycline and tetracycline) are bound to a similar extent by dog and human plasma and are unlike the sulfonamides, which are bound to a greater extent in human than in dog plasma(8). Our study also indicates that the order of increasing binding tendency is tetracycline → demethylchlortetracycline → chlortetracycline. These findings are in agreement with the work of Kunin *et al.*(2) and Kohn *et al.*(4). As to absolute binding value for each specific antibiotic, our results for binding of tetracycline by dog plasma are significantly lower than those (69% bound) reported by Pindell *et al.*(5). While studying renal excretion of tetracycline in the dog, Sullivan and Fanelli, of these laboratories, found that the Krebs-Ringer-bicarbonate buffer(9) gave higher binding values than the phosphate-saline buffer used in our study.

TABLE III. Binding of Tetracyclines at Various Concentrations in Human Plasma.

Drug	Drug conc. in plasma, µg/ml Total	 Unbound*	% bound
Tetracycline	2.16	1.34	38†
	1.84	1.31	29
	2.09	1.43	31
	6.10	4.07	33†
	6.02	4.42	26
			32 ± 4‡
Demethylchlortetracycline	1.13	.52	54†
	1.02	.47	54
	2.94	1.40	52†
	3.25	1.61	51
	3.05	1.58	48
	8.09	4.00	50†
	7.63	3.84	50
			51 ± 2
Chlortetracycline	1.37	.41	70†
	1.20	.48	60
	3.65	1.44	60†
	4.00	1.33	67
	4.12	1.51	63
	11.46	3.92	66†
	11.08	3.92	65
			64 ± 4

* $\frac{\text{g water/ml plasma}}{\text{g water/ml buffer}} \times$ µg drug/ml buffer = µg unbound drug/ml plasma.

† Drug was added to buffer except in these determinations where is was added to plasma.

‡ Mean ± S.D.

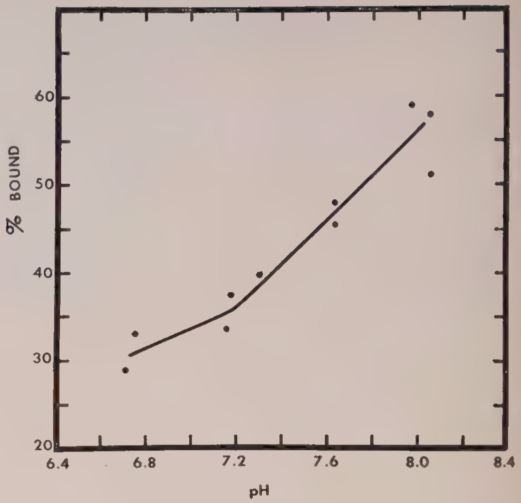

FIG. 1. Effect of pH on binding of tetracycline by dog plasma. Antibiotic concentration in plasma was 2-3 µg/ml.

Since the binding of drugs by plasma proteins is affected by concentration of drug in the plasma(10,11), protein concentration in the plasma(10), species,(11,12) pH,(10,13) temperature(10,14,15) and buffer, apparent disagreement in results from different investigators could be accounted for by variation in these conditions.

Summary. Dialysis equilibrium studies under physiological conditions of temperature and pH with tetracycline, demethylchlortetracycline and chlortetracycline have shown that degree of binding by dog and human plasma for drug concentrations ranging from 1-10 µg/ml was approximately twice as much for chlortetracycline (61%) as for tetracycline (34%), while demethylchlortetracycline was intermediate. These experiments also showed that each tetracycline was bound to a similar extent by dog and human plasma. A study with tetracycline showed that: a) approximately the same results were obtained when the equilibrium was carried out at 37°C or at 4°C; and b) binding increased with increasing alkalinity over the pH range of 6.7 to 8.1.

¶ Avg of 4 determinations. Ranges in parentheses.

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Received July 20, 1960. P.S.E.B.M., 1960, v105.

Zn^{65} Uptake by Rat Dorsolateral Prostate as Specific Bioassay for ICSH.* (26131)

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It has been demonstrated(1) that a measurement of the functional capacity of the rat dorsolateral prostate to concentrate Zn^{65} was a more sensitive indicator of ICSH (LH) activity than measurements of either dorsolateral or ventral prostate weight. The results of further studies to ascertain degree of sensitivity and specificity of Zn^{65} uptake as a bioassay for ICSH are reported in this paper.

Methods. Hypophysectomized (hypox) male Wistar rats (16-weeks of age, weighing 300 to 350 g) were purchased from Charles River Breeding Lab., Brookline, Mass. On the 5th day following surgery hormone injections were initiated as follows: 72 hypox rats received daily injections of 5, 20, 40, 100 and 200 μg of ICSH;[†] 52 hypox rats received daily injections of 200 and 500 μg of FSH,[†] Growth Hormone,[†] and Prolactin;[†] 12 hypox rats received daily injections of 20 and 40 μg of a human male castrate urine extract prepared and purified according to technics outlined by Albert(2). All hormone preparations were injected intramuscularly in 0.2 ml physiological saline, once daily for 6 days. Eighteen hypox rats injected with 0.2 ml physiological saline daily served as controls. On the 6th day of hormone administration all rats received intracardiac injections of Zn^{65} (0.04 μc/g) as outlined previously(3). Twenty-four hours later the rats were sacrificed, the dorsolateral prostates dissected(4), and their radioactive content determined by technics described(3). All rats used in this experiment were inspected grossly for completeness of hypophysectomy. None showed any pituitary remnants. During this investigation the rats were housed under constant

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* Supported by a grant from U. S. Atomic Energy Comm.

[†] Supplies of purified pituitary hormone preparations were generously furnished by the Endocrinology Study Section, Nat. Inst. Health; ICSH (NIH-LH-S-1, ovine), FSH (NIH-FSH-S-1, ovine), growth hormone (NIH-GH-B_2, bovine), and Prolactin (NIH-P-S-3, ovine).

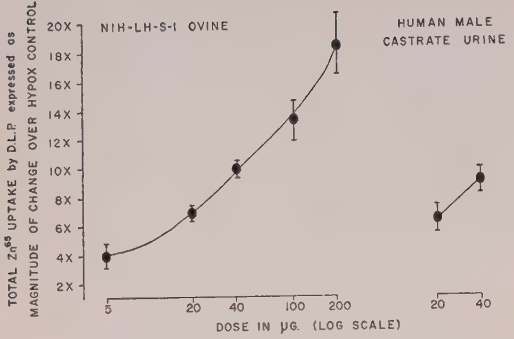

FIG. 1. Effect of various doses of ICSH and of purified male castrate urine extract on Zn^{65} uptake by dorsolateral prostate of hypophysectomized rat (mean ± S.E.M. are shown).

conditions of temperature, humidity and lighting (12-hour light, 12-hour darkness cycle). These experiments were conducted during the months of March, April and May, 1960(3,5,6).

Results. Zn^{65} uptake values, expressed as total counts per minute in dorsolateral prostate (mean ± S.E.M.), were as follows: for 18 untreated hypox controls, 2,500 ± 208; for 5 hypox rats administered 5 μg ICSH, 10,020 ± 2,380; for 18 hypox rats administered 20 μg ICSH, 17,100 ± 1,205; for 13 hypox rats administered 40 μg ICSH, 24,700 ± 1,730; for 10 hypox rats administered 100 μg ICSH, 32,900 ± 4,000; and for 16 hypox rats administered 200 μg ICSH, 46,330 ± 5,610 (Fig. 1). The data indicate that with the 5, 20, 40, 100 and 200 μg of ICSH there was a 4.0-, 6-8-, 9.9-, 13.2-, and 18.4-fold increase, respectively, over the untreated hypox control. Statistical analysis showed that with 5 μg of ICSH the Zn^{65} uptake response differed significantly from that of the untreated hypox control ($P < 0.005$). The response with 20 μg differed significantly from that with 5 μg ($P<0.01$) and from that with 40 μg ($P<0.001$).

Zn^{65} uptake values for the human male castrate urine extract were as follows: for 6 hypox rats administered 20 μg extract, 15,960 ± 2,900; for 6 hypox rats administered 40 μg extract, 22,440 ± 2,277. Fig. 1 shows that with 20 and 40 μg of extract there was a 6.4- and 9.0-fold increase in Zn^{65} uptake, respectively, over the untreated hypox control. Statistical analysis showed that with the low dose of 20 μg of extract the Zn^{65} uptake response differed significantly from that of the untreated hypox control ($P< 0.001$). Within the limits of error of the assay method, the human male castrate urine preparation was equipotent with the ICSH standard used, as judged from the parallel line multiple dose-response curves.

Zn^{65} uptake values in hypox rats administered 200 and 500 μg of Growth Hormone and of Prolactin did not differ significantly from that of the untreated hypox control. Zn^{65} uptake values in hypox rats administered 200 and 500 μg of FSH indicated the presence of approximately 1-2% ICSH activity.

Discussion. Previous investigations have shown that the amount of administered Zn^{65} taken up by the rat dorsolateral prostate depended upon androgen level(7,8). The functional capacity of the gland to take up Zn^{65} was diminished by removal of testes or pituitary, but could be maintained near control levels in the castrate by suitable doses of testosterone propionate(7) and in the hypox rat by maintenance doses of chorionic gonadotrophin or testosterone propionate(8). A more recent communication(1) demonstrated that with administration of 200 μg of ICSH to hypox rats total Zn^{65} uptake by the dorsolateral prostate was increased approximately 18-fold over that of the untreated hypox control, suggesting use of this technic as a possible bioassay for ICSH activity. In the experiments reported here it was demonstrated that doses of ICSH as low as 5 μg could be detected by virtue of a 4-fold increase in Zn^{65} uptake values over those noted in untreated hypox controls. That this method is suitable for a bioassay of clinical material was indicated by the fact that an extract of human male castrate urine gave a dose-response curve which was identical in slope with that of the standard ICSH preparation used.

These studies have also shown that the Zn^{65} uptake technic was a specific measure of ICSH activity as has also been claimed for the ventral prostate assay method(9). There was no significant response to either growth hormone or prolactin with doses as high as 200 and 500 μg. The presence of ICSH activity in the FSH preparation used is borne out by the reports of others.‡ The problem of LH or ICSH activity in preparations of FSH is a topic of much discussion(10). Woods and Simpson(11) have recently stated, "No follicle stimulant of natural origin (pituitaries of any species, menopause or castrate serum or urine) is known which did not at some relatively low multiple of the minimal dose for follicle-development also show uterine stimulation and luteinization or interstitial cell stimulation."

Summary. Experiments have been reported which indicate that a measurement of Zn^{65} uptake by the dorsolateral prostate of the hypophysectomized rat is a sensitive and specific bioassay for ICSH activity.

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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Endocrine Effects of Sulfoxone and of Two Nitriles as Compared with Amphenone.* (26132)

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Amphenone (3, 3-bis-(p-aminophenyl)-butanone-2), has been shown to exert marked endocrine effects on intact mammals through inhibition of formation of adrenal cortical and thyroid hormones by those glands and by a direct effect on the genital tract of the female(1). Because of the report of endocrine changes in patients treated with a sulfone(2) and because of the structural resemblance of the "parent sulfone" diaminodiphenylsulfone (DDS) to amphenone, it was decided to test the disodium formaldehyde sulfoxylate derivative of 4, 4′ diaminodiphenylsulfone (sulfoxone, "Diasone",® Abbott Pharmaceutical Co.) for such actions. Two steroid analogues bearing some structural resemblance to amphenone were also tested: 2, 3-bis-(p-hydroxyphenyl)-valeronitrile (SC-3402 of the G. D. Searle Co.), and 2, 3-bis-(p-hydroxyphenyl)-proprionitrile (Searle SC-4473). The formulae are given in Fig. 1.

Methods. The intact rats were immature females from 23 to 28 days of age obtained from the Holtzman Co., Madison, Wisc. Ovariectomies were performed on a group of these rats at this age one week before beginning drug administration. The hypophysectomized rats were adults of a Sprague-Dawley strain from the Hormone Assay Labs., Chicago, Ill. They had been hypophysectomized about 2 months prior

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* We would like to acknowledge the kind gifts of nitriles from G. D. Searle Co., Chicago, Ill.; sulfoxone from Abbott Labs., North Chicago, Ill.; and amphenone from Upjohn Co., Kalamazoo, Mich.

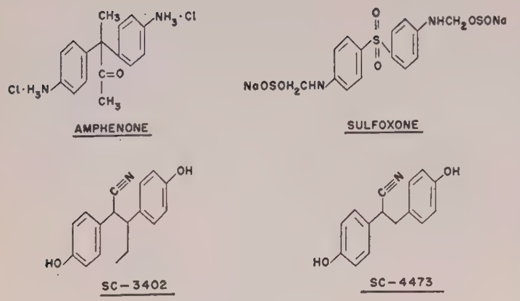

FIG. 1.

to beginning of experiment. The diet was Purina Lab Chow, and all controls were pair-fed. Experiments lasted 5 days each, except those with SC-4473 in the intact animals and in the ovariectomized animals which lasted 7 days. All substances were given in doses of 20 or 40 mg/100 g of body weight according to the weight as determined every other day. The sulfoxone and amphenone were administered in aqueous solutions by daily subcutaneous injections. SC-3402 and SC-4473 were suspended in corn oil and administered by stomach tube daily. Controls received the vehicles only. The day following the last day of experiment (the day after the 5th or 7th day of treatment) the animals were sacrificed, autopsied, and organ weights determined on a Roller-Smith torsion balance. Because of the close similarity of body weights in the test animals as compared with those of the controls, no allometric corrections were made. Statistical methods were those of Fisher(3). The sellar regions of the hypophysectomized animals were examined with a hand lens at autopsy for gross evidence of pituitary remnants, which were not found in any of the animals.

Results. Mean organ weights, expressed as mg/100 g of body weight, are presented in the table with standard deviations, an estimate of the significance of the differences between test and control values, and an expression of maximum change control value in per cent. As expected, administration of amphenone was followed by an increase in size of adrenals (68%), thyroids (77%), and uteri (62%), and by thymolysis and possibly a slight increase in ovarian size. None of the other substances showed an adrenomegalic effect similar to that seen with amphenone, and sulfoxone appeared inert by these measurements except for a decrease in ovarian and uterine weight on the lower dose, of no statistical significance. There were small changes in the ovarian, uterine, and thyroid weights of the SC-3402 animals. The SC-4473-treated rats showed marked enlargement of the uteri and ovaries with striking thymolysis. The uterine and thymic changes persisted in the hypophysectomized and the spayed groups.

Discussion. The absence of significant adrenal enlargement in the sulfoxone-treated rats is not surprising, considering that patients have been treated for leprosy for prolonged periods of time with this drug without signs of adrenal dysfunction. Possibly administration by the oral route might bring about some effects, since hydrolysis to the "parent sulfone" DDS is said to occur in the gastrointestinal tract. However, sulfones which are more active when administered orally may also be effective parenterally(4). While no adrenomegaly occurred with either of the nitriles, the marked changes in uterus and thymus, persisting in the hypophysectomized and ovariectomized animals, indicate that SC-4473 is markedly estrogenic. This property is of particular interest, since these represent the first nitriles to be shown to possess estrogenic activity. However, in our experiments the valeronitrile is inactive in contrast to the previous findings(5,6). This discrepancy might be due to species or strain differences, route or duration of administration, or dose, larger doses having been used in the present work. Uterine response in rats has not been previously reported for either nitrile. The adrenomegalic response previously noted was reported in intact male rats and was absent in hypophysectomized males suggesting that this may be similar to the response seen with other estrogens(7). The ovarian enlargement seen with SC-4473 also resembles the estrogenic effect seen in conjunction with gonadotropins and might be expected to disappear with larger doses, although uterine response was marked and the rats

TABLE I. Organ Weights as mg/100 g Animal.

	Dose, mg/100 g	No. animals	Mean body wt, g	Adrenals		Thyroid		Ovaries		Thymus		Uterus	
				Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Controls		5	79.6	35.9	3.6	9.0	1.8	53.9	12.0	369	45.4	123	33.0
Amphenone B	20	5	69.0	60.3	14.8	15.9	2.6	62.3	8.1	154	40.1	199	35.8
Max change				+68%†		+77%†		+16%		-58%‡		+62%‡	
Controls		10	91.0	33.0	2.3	7.5	.9	49.3	9.1	324	54.3	126	25.8
Sulfoxone	20	7	88.5	33.8	4.0	7.2	1.7	33.7	3.4	322	26.4	101	26.9
”	40	7	90.2	34.1	2.8	8.1	.6	41.4	9.0	292	78.7	129	41.7
Max change								-32%				-20%	
Controls		10	101.6	32.7	3.8	12.0	1.5	53.9	10.4	316	43.8	163	20.1
SC-3402	20	7	99.8	32.8	4.7	9.7	1.9	55.0	4.0	331	28.8	177	49.7
”	40	7	101.2	29.1	3.4	8.8	1.9	61.9	5.7	312	29.8	135	17.5
Max change						-27%†		+15%				-17%	
Controls		9	81.6	38.7	4.1	10.7	2.3	43.2	5.2	350	71.8	114	25.8
SC-4473	20	7	70.2	39.5	3.2	10.5	2.2	83.0	8.4	202	47.8	274	50.1
”	40	7	67.8	39.4	2.9	11.6	1.5	81.2	11.8	167	23.0	383	70.7
Max change								+92%‡		-52%‡		+236%‡	
Hypox controls		5	165.6	26.2	2.1	6.9	.8	36.2	7.1	162	51.8	85	13.0
” SC-4473	20	5	171.2	27.8	4.4	7.6	.9	39.3	5.9	120	35.6	267	113.5
Max change										-26%		+214%*	
Ovarx. controls		5	130.1	38.0	6.4	10.0	1.3			264	54.0	43.6	8.3
” SC-4473	20	5	121.3	36.9	10.1	9.0	1.1			250	51.3	110.6	27.4
” ”	40	5	127.7	35.0	4.9	8.5	1.5			209	39.8	157.0	70.8
Max change										-26%		+260%‡	

* P <.05 † P <.01 ‡ P <.001

quite young(8). An estrogenic response might be expected from the structural similarity of these compounds with the stilbestrols.

The reduction in thyroid weight seen with SC-3402 is unusual and may be related to the large thyroid size of the control animals in that particular lot. Likewise, the decreased ovarian and uterine weight in the rats on the lower dose of sulfoxone was not sustained with larger doses and is probably of no biological significance.

Summary. A sulfone, sulfoxone, and 2 nitrile steroid analogues, SC-3402 and SC-4473, have been tested in female rats for endocrine effects and compared with amphenone for reference. None of the 3 substances caused adrenal enlargement similar to that seen in the amphenone-treated animals. SC-4473 was markedly estrogenic. This substance represents the first nitrile to be shown to possess estrogenic activity in the rat.

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Received Sept. 1, 1960. P.S.E.B.M., 1960, v105.

Serum Thrombotic Accelerator (STA) Activity: Its Relation to the First Phase of Clotting.* (26133)

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Normal mammalian serum, in striking contrast to plasma, induces thrombosis in areas of vascular stasis essentially devoid of endothelial injury(1,2). The activity of the moiety in serum responsible for this phenomenon (serum thrombotic accelerator, or STA activity) is mediated through the intrinsic clotting system and is demonstrable only in serum prepared from blood initially rich in Hageman factor (HF), plasma thromboplastin antecedent (PTA), and Factor IX (PTC)(3). The present report further characterizes the relationship between STA activity and these factors.†

Methods and materials. STA activity of human and hen serum was measured in the rabbit by a previously described assay using a standard dose of 1.32 ml/kg(5). When STA activity was measured in the hen using hen and normal human serum, doses as high as 2.64 ml/kg were employed. The amount of thrombus formed was scored on a scale from 0 to 4; a score of 0 represented no clot; a score of 1, a few macroscopic strands of fibrin; a score of 2, several small thrombi; a score of 3, two or more large thrombi; and a score of 4, or complete thrombosis, represented a single thrombus forming a cast of the isolated segment(5).

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* This study was supported by research grants from Nat. Heart Inst., and by a grant-in-aid from Am. Heart Assn.

† Presented in part before Am. Physiol. Soc. April 12, 1960, Chicago, Ill.(4).

Serum preparations. Serum was prepared from blood collected by direct venipuncture from normal subjects with uncoated needles and syringes. The blood was allowed to clot in glass tubes for 2 hours at room temperature, and was then left for 18 hours at 4°C. The serum was separated by centrifugation at 2500 g at 4°C for 20 minutes and stored in glass containers at –20°C. Such serum is hereinafter referred to as "contact" serum. Serum was prepared as described for "contact" serum except that collection, preparation, storage, and infusion were carried out with silicone-coated equipment.‡ Such serum is hereinafter referred to as "intact" serum. The effect of surface activation of STA activity was investigated with 3 additional serum preparations. Twelve silicone-coated glass beads (0.5 cm in diameter) were added to 10 ml samples of blood collected with siliconized equipment and gently mixed by inversion in siliconized test tubes until a solid clot had formed. The clotted blood was thereafter allowed to stand for 2 hours at room temperature and handled as previously described. An additional series of blood samples was similarly processed except that 12 uncoated glass beads were substituted for the coated beads. Finally, serum prepared with silicone equipment was stored in uncoated glass tubes at –20°C for 24 hours. All 3 serum preparations were tested for STA activity by infusion into rabbits.

Aliquots of "contact" serum maintained at 20°C, 37°C, and 56°C for 15 or 20 minutes were also infused into rabbits. "Contact" serum was adsorbed with celite§ (30 mg/ml) for 20 minutes and centrifuged at 2500 g and 4°C for 20 minutes to remove the celite powder. "Contact" serum adsorbed with chemically pure barium sulfate|| (100 mg/ml) for 20 minutes was similarly prepared. The barium sulfate adsorbate was recovered by elution with sodium citrate as previously described. All 3 serum fractions were infused into rabbits at the standard dose(5). Decalcified human serum was prepared by addition of 1 volume of 0.1 M sodium oxalate, 1 volume of 0.1 M sodium citrate, or 1 volume of 0.29 M of disodium ethylenediaminetetraacetic acid (EDTA) to 9 volumes of contact serum. Hen serum was prepared from blood obtained by cardiac puncture according to the procedure used for "contact" human serum. Platelet-poor plasma(6) was infused into rabbits at the standard dose (1.32 ml/kg). Platelet-poor plasma, recalcified with equal parts of 0.025 M calcium chloride, was also infused into rabbits at a dose of 2.64 ml/kg after the fibrin clot had been expressed with a wooden applicator.

TABLE I. Effect of Temperature, Adsorption, Calcium, and Platelets on Human STA Activity.

Serum treatment	No. of rabbit infusions	Complete thrombosis (% animals)
Temperature ("contact" serum)		
20°C/20 min.	7	100
37°C/20 "	7	100
56°C/15 "	5	0
Fractionation ("contact" serum)		
Celite adsorption	4	100
$BaSO_4$ "	6	0
Sodium citrate eluate after $BaSO_4$ adsorption	6	100
Decalcification ("contact" serum)		
.1 M sodium citrate	2	100
.1 M " oxalate	2	100
.29 M EDTA	2	100
Plasma		
Platelet-rich (>300,000 cells/mm^3)	6	0
Platelet-poor (<100 cells/mm^3)	3	0
Recalcified platelet-poor plasma	3	100

Results. Table I shows assays of STA activity of human serum subjected to various physical and chemical modifications. Serum maintained at 20°C or at 37°C for 20 minutes contains full STA activity, whereas similar aliquots heated to 56°C for 15 minutes, lose this activity. "Celite" adsorbed serum exhibits complete STA activity in contrast to barium sulfate adsorbed serum. Two infusions each of serum decalcified with sodium oxalate, sodium citrate,

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‡ Siliclad, Clay-Adams, Inc., New York; Monocote-E, Armour Lab., Kankakee, Ill.

§ Johns Manville, Inc., Boston, Mass.

|| Bakers, Phillipsburg, N. J.

TABLE II. Relative Roles of HF, PTA, and Factor IX in Donor Serum and Recipient Animal.

Donor	Recipient	No. of infusions	Complete thrombosis (% animals)
Hen	Hen	8	0
"	Rabbit	6	0
Human	Hen	11	64
"	Rabbit	6	100

and EDTA were all capable of inducing thrombosis in rabbits. Finally, platelet-poor plasma lacks STA activity, whereas when recalcified this activity is demonstrable.

Hen serum is incapable of inducing thrombosis in hens or rabbits, whereas human sera can initiate intravascular coagulation in the hen as well as the rabbit (Table II). Of 11 human samples infused into the hen, 7 infusions yielded a score 4 thrombus; 2, a score 3 thrombus; 1, a score 2 thrombus; and 1 a score 0 thrombus. Thus, more than 90% of these infusions of human serum into hens demonstrated partial or full STA activity.

The effects of "surface" on STA activity of normal human serum are shown in Table III. STA activity is not demonstrable in "intact" serum in contrast to "contact" serum. It is evident from the 38 serum samples tested in the rabbit assay that activation of STA is dependent on exposure of "intact" serum to "foreign" surface.

Discussion. Previous studies have demonstrated that Factors I (fibrinogen), II (prothrombin), III (thromboplastin), V (ac-globulin), VII (convertin), VIII (antihemophilic factor), and X (Stuart factor) are not required in the donor serum for STA activity to be manifest(1,3,4). Results of infusions with recalcified platelet-poor plasma indicate that platelets, too, are not required for the elaboration of STA activity (Table I). Whereas HF, PTA, Factor IX, and possibly Factor IV (Ca^{++}) are essential for STA activation, the data in Table I indicate that STA is distinct from HF, PTA, and Ca^{++}. HF and PTA activities are relatively stable in serum at temperatures as high as 60°C(7,8). In contrast, STA activity is lost under these conditions. Moreover, barium sulfate adsorbed serum, rich in HF and PTA(7,9) cannot induce a thrombus, whereas the sodium citrate eluate of the barium sulfate adsorbate, relatively devoid of these 2 factors, can cause thrombus formation. In addition, "celite" adsorbed serum, also devoid of HF and PTA (10) retains the capacity to induce thrombi.

The hen, congenitally lacking in HF, PTA, and Factor IX(11) provides an opportunity to determine the relative roles of these 3 factors in donor serum and recipient animal. The data in Table II are consistent with previous observations(3) that these 3 coagulation factors are required for activation of STA in the donor serum and it is also clear that HF, PTA, and Factor IX need not be present, even in an inactive form, in the recipient animal for thrombus formation to occur.

Although STA activity can be distinguished from HF and PTA, no fractions in normal human serum have yet been prepared in which STA activity has been obtained free of Factor IX. The 2 serum fractions (Table I) exhibiting STA activity also contain Factor IX. Barium sulfate adsorbed serum, devoid of STA activity, lacks Factor IX. Serum prepared from patients

TABLE III. Effect of Surface on STA Activity of Human Serum.

Collection	Preparation	Storage	Infusion	No. of infusions	Complete thrombosis (% animals)
Glass*	Glass	Glass	Glass	6	100
Silicone	Silicone	"	Silicone	8	62
"	Silicone and glass beads	Silicone	"	6	60
"	Silicone and siliconized beads	"	"	9	11
" †	Silicone	"	"	9	0

* Contact serum. † Intact serum.

congenitally deficient in Factor IX exhibit no STA activity despite normal amounts of HF and PTA. This might suggest that the 2 entities, STA and Factor IX, are identical. However, in a study of the influence of Dicumarol on STA activity in dogs, it was shown that loss of this thrombotic activity was dissociated from the depression of any coagulation factor known to be reduced by Dicumarol singly, or in combination(12). Although a temporal correlation between loss of STA activity and depression of Factor IX occurred, a clear-cut dissociation between STA activity and Factor IX was, nevertheless, observed(12). Similar studies, currently in progress in man, show comparable results and indicate that STA and Factor IX may not be identical.

Even though STA activity is distinct from HF, the experiments with various types of "contact" and "intact" serum clearly indicate that elaboration of STA activity is dependent, in part, on prior activation of HF. Failure to achieve complete activation with uncoated glass beads may be the result of inadequate glass surface area. Similarly, the inability to prevent partial activation with siliconized glass beads may be attributed to incomplete silicone coating of the beads. These results may reflect the effect of exposed, uncoated surface on activation of HF.

Several investigators, using *in vitro* technics, have concluded that HF and PTA are the initial reactants in the sequential steps whereby intrinsic thromboplastin is elaborated(13,14,15), and that the "complex" formed by these 2 factors activates Factor IX before reacting with platelets or with any other coagulation factor involved in the first stage of clotting with the possible exception of Ca^{++}(15). Whether Ca^{++} is required in either or both of these steps *in vitro* has not yet been established(16). The data obtained in our laboratory, using *in vivo* thrombus formation as the endpoint, are consistent with these interpretations of *in vitro* observations. The findings with various types of "contact" and "intact" human serum emphasize the pivotal role of "surface" and HF activation in the elaboration of STA activity. It has also been observed in *in vitro* experiments that activation of HF can result in further activation of the components of intrinsic thromboplastin in absence of PTA. In contrast, no activation can be demonstrated when HF is absent(14). This finding provides a possible explanation for our earlier results wherein HF-deficient serum was essentially devoid of STA activity but PTA-deficient serum exhibited a significant though less than normal amount of STA activity(3).

From the viewpoint of the pathogenesis of thrombosis, STA represents a potent thrombotic activity, appearing early in the elaboration of intrinsic thromboplastin, distinct from platelets, tissue thromboplastin and thrombin and inducing a thrombus that initially is histologically dissimilar from that produced by tissue injury(2). The relevance of these observations to initiation of spontaneous intravascular thrombosis will depend, in part, upon elucidation of the role of endothelial surface in the activation of Hageman factor.

Summary. Serum thrombotic accelerator (STA) activity requires for its elaboration activation of HF, PTA, and Factor IX, but does not depend on platelets or any other recognized coagulation factor with the possible exception of Ca^{++}. Although essential in the donor serum for formation of this thrombotic moiety, HF, PTA, and Factor IX need not be present in the recipient animal for thrombus formation to occur. STA activity is distinct from Ca^{++}, HF, and PTA, and may not be identical with Factor IX; however, activation of HF appears to be the initial reaction required in the elaboration of this potent thrombotic agent that is distinct from thrombin and tissue thromboplastin.

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Received Sept. 9, 1960. P.S.E.B.M., 1960, v105.